

California dreaming

Voters in California will decide next month on an initiative that would assign \$3 billion to research on human embryonic stem cells. But the proposal is less of an unalloyed blessing than it seems.

Research on human embryonic stem cells has become a bone of fierce political contention in the United States. President George Bush's decision to allow federal funding only for research on stem-cell lines derived before 9 August 2001 has been attacked by his rival, Senator John Kerry, most recently during last week's second presidential debate.

In California, the policy has drawn an even more direct response from the biotechnology industry and parts of the research community. 'Proposition 71', on which California's citizens will vote on 2 November, would enable a bond issue that pumps \$300 million a year for ten years into research on human embryonic stem cells. The proposition would establish an institute to review grant applications and distribute the funds, and would insulate it from the state legislature, so the money could not be redirected by political whim. The initiative would even amend the state constitution to guarantee the right to do embryo research.

Many researchers in California see this as a chance to flex some political muscle. At first glance, the proposition would close an unfortunate gap in federal research funding and instantly transform the state into a hub of activity in a promising field. But aspects of the proposal should give voters pause before they lend it their support.

First, it isn't clear that this particular field of study can make the best use of the substantial amount of money that the proposition plans to throw at it. In a manner pioneered by campaigners for public funding for sports stadia, the proposition's advocates have delivered a series of "economic analyses" arguing that the bond issue will pay for itself in the long run, by nurturing economic growth through the development of the local biotechnology industry. But it is not clear that these analyses hold water. And the vote is taking place after

a series of other ballot-driven commitments to low taxes and high spending, which have driven California to near-insolvency.

The exclusion of the state legislature from responsibility for overseeing the programme is a further cause for concern. Whatever one thinks of individual politicians, democratic supervision places important constraints on the use of public money. The federal agencies that fund most academic research in the United States — the National Institutes of Health (NIH) and the National Science Foundation — operate under the scrutiny of Congress. At these agencies, scientific merit is judged almost entirely by the community itself, but Congress ultimately ensures that the public good is paramount.

Proposition 71, in contrast, would introduce a new model for the support of scientific research at the state level that would rely on mere transparency as a guarantee against abuse. Although public meetings are promised, the oversight committee would consist mainly of people with close ties to the universities, institutes and companies that stand to benefit from the money spent. Most of the rest are representatives of disease groups. The committee makes the ultimate funding decisions and will be allowed to modify NIH rules of informed consent and human-subject protection as it sees fit.

The advocacy of such people as the actor Christopher Reeve — whose untimely death this week deprives biomedical research of one of its most forceful and effective lobbyists — has helped to elevate the promise of embryonic-stem-cell research, sometimes to unrealistic levels. It is up to the people of California whether they want to approve Proposition 71. But if they do, researchers must strive to ensure that no funds will be abused, and they must give full consideration to a wide array of ethical concerns. Anything less risks damaging public trust in science. ■

Against grade inflation

How to counter declining rigour in US university courses.

Call it Moore's law of US higher education: the quantity and quality of work that undergraduates must do to get top grades halves every decade. This is an exaggeration, of course, but many readers will recognize the sentiment. Is it just the jaded perception of cynical academics? On the contrary: the evidence suggests that there is a real problem of grade inflation in degree courses, especially at private universities. And the assessment of teachers by students, as well as parents' demands that they get what they think they've paid for, are making the problem worse.

Course evaluations were intended to give the instructor feedback about how well he or she was doing. But they rapidly became a favoured tool of deans, tenure and promotion committees because they were quantifiable. Now there is an implicit understanding that if instructors give good grades, they will not be judged too severely by students. New faculty often grade more harshly than other members of the department, only to be 'punished' by students. Deans who believe that this doesn't happen are deluding themselves.

Also worrying is the idea — particularly evident at costly private

universities — that students and their parents believe they are paying for a degree that will lead to a good job, rather than for a good education that will help them to think independently. The pressure on teachers to appease demanding students and parents by awarding high grades is obvious.

The consequences are all too clear. Anecdotal evidence suggests that there is a general unwarranted upward creep in grades (<http://ctl.stanford.edu/Tomprof/index.shtml>). More objectively, the fact that graduate schools rely for admission criteria almost exclusively on the results of standardized tests, rather than on universities' individual grading, points to a systematic failure to ensure that grading standards are being maintained.

What to do? More universities should focus seriously on improving the instructional abilities of their faculty in programmes — mandatory for new instructors — to videotape classes and analyse them with the faculty member to highlight strengths and weaknesses. And evaluations should take note of thoughtful individual comments by students, rather than relying on scores, or be abandoned. ■





Feeling the pinch
United States has
no substitute for
lost flu vaccine
p726



Off course
Ecologists grouse
over construction
of Black Sea canal
p727



Post palaver
Science advisers
quit over selection
of museum head
p728



Green Peace
Wangari Maathai
reaps Nobel for
grass-roots work
p730

Universities fear repercussions as NIH tunes conflicts policy

Erika Check, Washington

A high-profile investigation into conflict-of-interest policies at the National Institutes of Health (NIH) is having an impact way beyond Washington, as universities around the United States work out how it might affect their medical research centres.

So far, the congressional investigation has focused on policies governing the conduct of the 6,000 intramural scientists who work directly for the agency, mostly at its main campus in the Washington suburb of Bethesda, Maryland.

And investigators have not publicly announced that they plan to extend the investigation to university medical centres, where 200,000 scientists at all levels are supported by almost \$20 billion in annual funding from the NIH (see chart). But an official at the agency, who did not want to be named, says that questions about the universities' conflict-of-interest policies have already been raised by the investigators. And campus officials are tracking the subject closely, because Congress is clearly concerned about the issue and NIH policy changes are likely to affect universities, directly or indirectly.

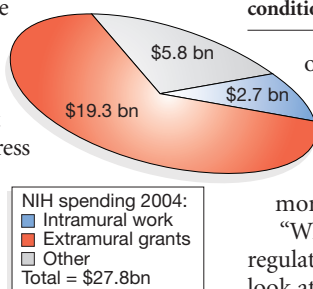
"It matters that Congress is taking an interest," says Julie Gottlieb, assistant dean for policy coordination at the Johns Hopkins University School of Medicine in Baltimore, Maryland. "It will result in changes at NIH, and it may signal changes for NIH grantee institutions."

The agency has already said it will change its own policies, requiring more disclosure and reducing the amount of paid consulting that its senior scientists are permitted to do. It is also planning to enact a one-year moratorium on paid consulting for all of its scientists (see *Nature* 431, 497; 2004).

This moratorium is more drastic than any measure a university is likely to contemplate. Still, such changes could affect universities in many ways. When the NIH's rules



Side-effects: revisions to the National Institutes of Health's conflict-of-interest policy could affect conditions on the outside grants that account for three-quarters of the agency's spending.



on conflicts of interest are finalized, for example, the agency could decide to create new rules for grantees. Or universities could choose to follow the lead and adopt rules that are more in line with those of the agency.

"When you see the body responsible for regulation changing its own rules, you have to look at its changes," says Theresa Colechia, associate general counsel at the University of Pittsburgh. "It can be an indication of where those grant-funding agencies will go with regulations for grant recipients in the future."

Policies strengthened

According to a survey released last month by the Association of American Medical Colleges (AAMC), most medical schools have adopted conflict-of-interest policies for faculty involved in human research. And 63% of these schools have strengthened their policies since 2001. Although the Public Health Service, the division of the federal health department that includes the NIH, issues broad guidelines for these policies, the specifics vary across universities.

For instance, 40% of medical schools surveyed by the AAMC do not require faculty

members to disclose potential financial conflicts in verbal presentations. Three-quarters of the schools have set up standing committees to evaluate conflicts of interest, but only 21% of these schools require community representation on the committee. And just slightly more than half of the schools surveyed require the standing committees to review technology-licensing agreements that may influence future research undertaken by individual faculty members.

Most university officials say that the policies at their own institutions are sound and that they are not planning alterations. "We have one of the broadest conflict-of-interest policies of any institution," says Barbara Flynn, manager of the conflict-of-interest review programme at Stanford University School of Medicine in California. "I don't think there's anything more we can do."

For the moment, US universities are not in line for a direct grilling by Congress: staff members on the committee leading the NIH investigation say they do not plan to spread their net to campuses. But university officials will not rest easy until the investigation ends, and the NIH rules are set.

GETTY IMAGES

SOURCE: NIH

US lacks back-up for flu vaccine shortfall

Helen Pearson, New York

Public-health officials in the United States were left scrambling for alternative sources of influenza vaccine last week, after the surprise announcement that half the country's supply for this winter had been shelved.

The problem came to a head on 5 October, when British regulators suspended the production of flu vaccine at the California-based Chiron Corporation's facility in Liverpool, UK, because of concerns over bacterial contamination. The suspension jeopardizes around half of the US supply.

In Britain, where about 20% of this year's requirement was to come from the Liverpool plant, public-health officials have been able to arrange alternative supplies. But the US government says that it cannot do the same.

In an emergency hearing of the government reform committee of the House of Representatives on 8 October, vaccine manufacturers, congressmen and public-health officials blamed each other for the lack of a contingency plan. "We need a surge capacity," said Julie Gerberding, director of the Centers for Disease Control and Prevention in Atlanta, Georgia, "and we simply do not have it."

The episode highlights the vulnerability of the current influenza vaccine manufacturing process, says infectious-disease specialist Donald Burke at Johns Hopkins Bloomberg School of Public Health in Baltimore. "It's a frustration to all of us that the system is as fragile as it is."

Flu vaccines are mass-produced by growing the virus in fertilized hen's eggs. About one egg is needed per dose, so vast numbers are ordered from specially bred hens as much as



Making sure: Americans are getting their flu jabs early this year as a vaccine shortage looms.

three years before production begins.

Each egg is injected with a tiny dose of the flu virus selected for the current year's jab. The virus grows for several days in the fluid surrounding the embryo before the eggs are sliced open, the liquid sucked out, and the virus extracted from the fluid and inactivated. The entire process, including safety tests on the vaccine, takes five to six months.

Egg-based production is tried, tested and reliable — but cannot manufacture vaccine at short notice. One way to bypass eggs is to produce the virus in mammalian cell cultures, which can be set up more quickly. The virus multiplies in the cells and is then filtered out of the reactor.

Brussels-based Solvay Pharmaceuticals has built a production plant for making flu vaccine in cell culture, and hopes that the first batches will be licensed and available by 2006. But the process has not taken off widely because most companies are reluctant to invest in expensive new equipment — and vaccines produced this way will still have lengthy safety testing and regulatory approval.

Even if production time can be squeezed, critics say that flu vaccine supply is vulnerable because only three companies are licensed to produce it for the US market. Many pharmaceutical companies are dissuaded from making vaccines because they return relatively little profit compared with drugs. ■

Californians up in arms over water assessment

Emma Marris, Washington

California legislators are demanding an inquiry into allegations that a local office of the National Oceanic and Atmospheric Administration (NOAA) reversed the findings of its own biologists in assessing a water-management plan for the state.

An early version of the plan, which *Nature* was shown, states that a strategy for managing the estuary around San Francisco Bay is "likely to jeopardize the continued existence" of two subspecies of fish, the Sacramento River winter-run chinook salmon and the Central Valley steelhead.

But a later version of the plan — dated September 2004 — comes to the opposite conclusion, stating that it "is not likely to result in jeopardy to the continued existence" of these fish.

Environmentalists claim that the first

version of the document was prepared by NOAA biologists in the Sacramento area office and that staff at NOAA Fisheries Southwest Regional Office in Long Beach, California, then rewrote it, with input from the US Bureau of Reclamation, a branch of the interior department, which drew up the water-management plan in the first place. Their claims were first reported in the *Sacramento Bee* on 2 October.

Bill Jennings, head of the watchdog group Deltakeeper, calls the revision "an outrageous sacrifice of sound science on the altar of expediency, that will lead to the further degradation of fisheries throughout the Central Valley".

But Jim Lecky, a marine biologist and NOAA official charged with preparing the final report, says that the original version failed to take account of recent adjustments

to the water-management plan. "It's much ado about nothing," he says. "There were some key errors made by the staff. It's not a matter of changing the science."

Last week, California state senator Michael Machado called for an independent review of the process. And in Washington, Congressman George Miller (Democrat, California) and 18 other members asked the inspector generals at the interior department and the commerce department — of which NOAA is part — to investigate.

Lecky says that a final version of the assessment will be published in a few weeks. ■

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Ukraine deluged by protests over plans for Danube delta

Quirin Schiermeier, Munich

Ukraine is coming under growing international pressure to halt the construction of a shipping canal through the ecologically sensitive Danube delta. Critics say the project will damage some important fish and wetland bird habitats, and increase erosion on the Black Sea coast.

Yet despite a mass of protests from scientists, environmentalists and national governments, work continues on the site. Protesters say the only concession made by Ukraine so far is a promise last week to open lines of communication to concerned parties.

The Danube delta is no stranger to environmental havoc. In the 1980s, Nicolae Ceausescu, Romania's communist dictator, disastrously tried to transform large parts of the wetland into arable land. Since his execution in 1989 the area has recovered — but environmentalists say it is again under threat.

The Ukrainian government is in the middle of a €30-million (US\$37-million) plan to expand a natural waterway called the Bystroye Canal, which connects the outflow of the River Danube to the Black Sea. This waterway was once used for shipping, but its ports closed after the 1994 Balkan war. Since then the waterway has filled with silt, causing the economy of the region also to stagnate.

By August, a 3-kilometre stretch of the waterway had been dug, making it passable for large cargo ships once more.

Work is expected to continue on 170 kilometres of the channel until 2007; it will then have to be continually dredged to stay navigable.

Ukrainian authorities say the project carries no serious environmental risks. But ecologists fear that continuing the work will destroy bird and fish habitats. The delta is currently home to some 300 bird species, including many rare birds such as white pelicans and pygmy cormorants. The labyrinth of waterways, lakes and dunes was declared a World Heritage site by the United Nations in 1991, and was made a Wetland of International Importance under the Ramsar Convention on Wetlands in 1995.

"We want to clearly state that the convention does not endorse the Bystroye project in its current design," says David Bridgewater, secretary-general of the Ramsar convention, based in Gland, Switzerland. "We are deeply



The right course? A 3-km stretch (boxed) of waterway to the Black Sea has already been dug.

concerned about Ukraine's repeated deafness to the recommendations of the international community," he says.

Although critics acknowledge that the impoverished region needs a new port, they say there are better places to carve a canal.

In the past few months, the United States and the European Union have officially asked Ukraine to stop all construction work pending an independent scientific assessment.



Delta blues: a canal expansion may oust white pelicans.

Following a visit from the European Commission and Ramsar convention delegates last week, Ukraine has promised to increase communication with those concerned about the project. Deputy transport minister Valentin Kasapchuk

says the country will convene an international meeting of experts in November to examine the likely impact of the project's next phase. He promises that there will be further monitoring of the environmental impact before construction continues.

Scientists and environmental groups hope to bring to officials' attention more than concerns about biodiversity. Geologists, for example, warn that the expansion will dramatically increase discharge from the Danube through the channel, depriving other parts of the delta of water, and causing coastal erosion.

Liviu Giosan, a marine geologist at Woods Hole Oceanographic Institution in Massachusetts, plans to use computer models to simulate the potential changes in the delta so as to better assess the impact of the project. "It's about time we stopped gambling with the future of a natural paradise," says Giosan. ■

Canada to join 'big league' with its own science academy

David Spurgeon, Montreal

Rectifying an omission that has irked its scientists for decades, the Canadian government is to mandate the creation of a Canadian Academies of Science.

The mandate, announced on 5 October by Prime Minister Paul Martin, will create what one adviser calls "an exact analogue" of the US National Academies, albeit on a smaller scale.

The academy will operate at arm's length from government, Martin said, although it will receive Can\$35 million (US\$28 million) of government funding over the next ten years. The arrangement will provide "a source of expert advice on scientific aspects of important domestic and international issues", he added.

For years, the Royal Society of Canada has performed one-off studies of issues for government departments, through ad hoc panels operating under the rubric of the Canadian Academy of the Sciences and Humanities. But in contrast to the situation in the United States, the society operated without either a professional staff or a formal government mandate.

In 2002, the government's industry department announced plans to create a proper Canadian science academy (see *Nature* 415, 824; 2002), but until this year nothing had been done to implement them.

William Leiss, a political scientist at Queen's University in Kingston, Ontario, and a past president of the Royal Society, has been advising the government on the proposed academy. He says Martin's announcement "will allow us to play with the boys in the big leagues".

Leiss was also involved in setting up the previous one-off studies. These included a controversial 2001 investigation of genetically modified foods (see *Nature* 409, 749; 2001). But he says such studies were "very tricky" to undertake, because their reliance on government grants made it hard for them to appear truly independent.

For many scientists and policy specialists in Canada, the foundation of a proper academy structure is decades overdue. "It should have been done a long time ago," says Leiss. "The US National Academies have original legislation going back to 1863 and the Royal Society in Britain has had a relationship with the British government since 1849." Even smaller countries such as the Netherlands and Austria have established similar bodies, he says. "It's been terribly frustrating for us." ■

Advisers rebel over choice of museum chief

Alison Abbott, Munich

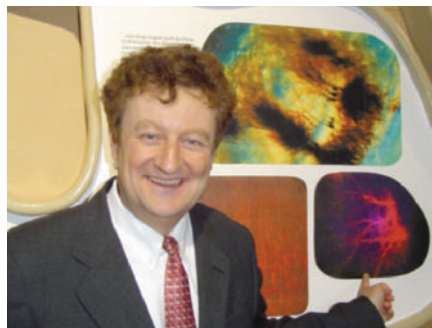
The head of the scientific advisory board of the Deutsches Museum — one of the world's largest science museums — has resigned in protest at the selection procedure for the museum's new director.

Jürgen Renn, chairman of the board and a director at the Max Planck Institute for the History of Science in Berlin, says the museum's administrative council ignored the board's input when it made the appointment in August. Two other board members have resigned, and the remaining seven have expressed concerns about the selection process.

Board members stress that they have no quarrel with Wolfgang Heckl, the physicist who took up the director's position on 1 October (see *Nature* **431**, 612; 2004). Renn says, for example, that Heckl "certainly has the communication skills that are needed to run the museum".

But they say that the administrative council failed to seek their opinion when making its decision. "The administrative council took no account of other factors required for leadership of such a museum — particularly experience with museums or the history of science," Renn says. He adds that the council did not make its selection methods and criteria clear.

Wolfgang Herrmann, president of the Technical University of Munich and head of the administrative council, says the procedure was "as transparent as it needed to be". The administrative council is solely responsible for selecting the candidate, and



Wolfgang Heckl: his appointment as director of Munich's science museum has raised hackles.

was glad to receive input from its scientific advisory board. "But the scientific panel had a different opinion to that of the administrative council — this happens sometimes," says Herrmann.

Paolo Galluzzi, a board member and head of the Science Museum in Florence, says he supports Renn's decision to quit. Galluzzi says he decided not to resign himself because



Deutsches Museum: consolidating its research.

he "doesn't want to weaken the scientific advisory structure further". But he adds that the remaining panel members are prepared to resign unless they receive assurances from the administrative council that their advice will be listened to in future.

Jochen Brüning, another board member and a mathematician at Humboldt University in Berlin, where he curates a museum collection, says he wants to give the new director as much support as possible. He adds: "It would be tragic if some new way was not found to have an effective external scientific advisory structure."

Most of the €33 million (US\$40 million) in annual funding for the Deutsches Museum comes from the Bavarian government and the German federal government. Almost one-third of it is spent on research.

The museum fared badly, however, in a 1998 external evaluation. Helmut Trischler, a science historian and the museum's head of research, says that the museum has been steadily consolidating its research since then, and won a strong endorsement in a 2002 evaluation. "The scientific advisory board worked hard in helping us root research more strongly within the museum as a whole, and we have implemented some of their suggestions," says Trischler.

Heckl says he is disturbed by the furore over his appointment, but reassured by the expressions of personal goodwill from the scientific advisory board. He points out that he does have experience of running exhibitions — one of them was shown in the Deutsches Museum itself in 2001. ■

Paris collections snubbed in spending review

Sally Goodman, Paris

Researchers at France's National Museum of Natural History are growing worried about the future of their collections, after this summer's government spending review failed to earmark any money to maintain them.

In July, France's research and environment ministries agreed to increase their joint annual contribution to the Paris museum's upkeep from €12 million (US\$15 million) to €19 million. But they did not include any cash for the collections, which — with around 65 million specimens — are among the most extensive in the world.

Museum staff claim that the omission occurred because the government's scientific advisers do not value taxonomy, or understand its importance to biodiversity research. President Jacques Chirac, however, has identified biodiversity as one of his top

research priorities, and is backing an international meeting on the subject in Paris next January.

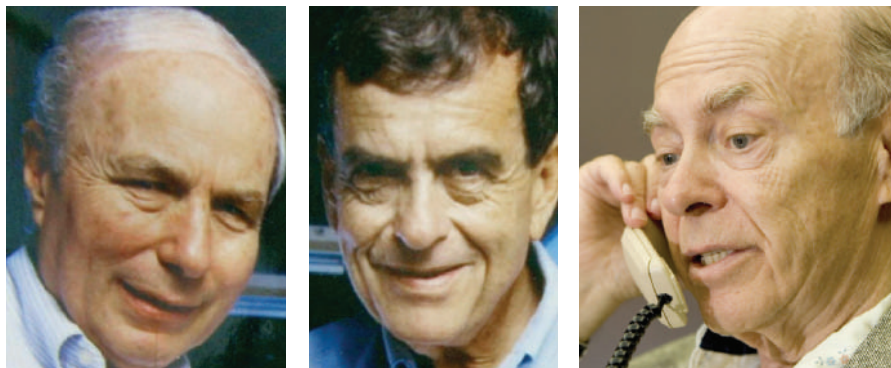
The lack of funding for the collections is "scandalous", complains Simon Tillier, head of the museum's department of systematic biology and evolution.

Despite the lack of government backing, the museum's director, Bertrand-Pierre Galey, says he plans to develop the collections, starting next year with the modernization of the world's largest herbarium. There are also plans to move the palaeontology collection from its current basement home, where it is at risk of flooding, and to continue a project to digitally catalogue the collections. But the museum will now have to fund these projects at the expense of other activities — such as research — or else find private backing for them.

Staff at the museum say that morale has been improving of late, after a restructuring reduced the power of senior professors and reorganized the museum's 26 laboratories into 7 research departments. But researchers are complaining that a lack of resources for maintaining the collections will render specimens difficult to access and vulnerable to deterioration.

Yvon Le Maho of the Paris museum's scientific advisory board believes the situation reflects a growing rift in modern biology. "The study of whole organisms and populations is seen as second-rate science compared with molecular biology, but you cannot dissociate the two," he says.

The Parisian curators say they are hoping for more support for their collections from the government when it reviews the museum's operating contract in two years' time. ■



All for one, from left: Hershko, Ciechanover and Rose worked together on the ubiquitin kiss of death.

Chemistry Nobel for trio who revealed molecular death-tag

Jim Giles

Avram Hershko thought he might have a chance of winning this year's Nobel prize — for medicine. So when the results were announced last week, and his name wasn't on the list, Hershko decided to spend some time by the pool with his granddaughters: "Maybe some other year, I thought."

But for Hershko, a biochemist at the Israel Institute of Technology in Haifa, it turned out to be the right year after all. While he was enjoying his holiday, Israeli radio announced on 6 October that he was a co-winner of this year's chemistry Nobel prize, together with Aaron Ciechanover, also at Haifa, and Irwin Rose of the University of California, Irvine. Hershko found out when his cousin phoned and told him.

The trio was rewarded for unravelling the mechanism behind a molecular kiss of death — a tag that marks proteins for destruction. The finding sparked new ideas about how cells regulate themselves, and spurred new directions in cancer research. "Their work is seminal," says Paolo di Fiore, a biochemist at the FIRC Institute of Molecular Oncology in Milan, Italy. "It affects every aspect of cell biology."

The laureates revealed the molecule that serves as this tag in two pioneering papers (A. Ciechanover *et al. Proc. Natl Acad. Sci. USA* 77, 1365–1368; 1980 and A. Hershko *et al. Proc. Natl Acad. Sci. USA* 77, 1783–1786; 1980). They later realized that the molecule was ubiquitin — a small protein that had been identified in 1975 by other researchers, but whose function had not previously been known.

Ubiquitin molecules can be found in many parts of the body — hence its name, taken from the Latin for 'everywhere'. Following the prizewinning discovery, biochemists now know that proteins are tagged with several ubiquitin molecules before being shuttled to the proteasome, a barrel-shaped structure that chops up proteins.

"Once the pattern was clear, work blossomed into a huge number of areas," says Jeremy Berg, director of the National Institute of General Medical Sciences in Bethesda, Maryland.

Cancer researchers, for example, subsequently learned that ubiquitin regulates the levels of the protein p53, dubbed the 'guardian of the genome' for its role in protecting against cancer.

In a normal cell, ubiquitin regularly binds to p53 proteins, shunting them off for destruction in the proteasome. In cells where the DNA has been damaged, ubiquitin does not bind to p53, allowing levels of the protein to build up. This, in turn, stops cell division, allowing DNA repair systems get to work, or leading to the death of the cell if the damage is too great. In many tumours, this safety-control system has been disabled, allowing mutant cells to survive and multiply. This realization has led to the development of anticancer drugs that mimic this system to halt cell division.

Another contributor to this work on protein degradation was Alexander Varshavsky, a cell biologist at the California Institute of Technology in Pasadena. In 2000, Varshavsky shared the prestigious Albert Lasker Award for Basic Medical Research with Ciechanover and Hershko for work on ubiquitin. But he is absent from the list of Nobel winners.

Berg suggests that this may be because Varshavsky's work, which confirmed theories about ubiquitin by studying its action in yeast, may have been judged to be closer to cell biology than biochemistry — making it a step too far removed for a chemistry prize. Rose, in contrast, laid the groundwork for the 1980 papers by studying the chemistry that underlies the ubiquitin pathway.

"I'm very, very sorry for Varshavsky," says Hershko. "He really deserves it. The importance of what we had done only became clear after Varshavsky's work. I'd be happier if there could be four winners."

'Memory of water' biologist dies after heart surgery

Philip Ball, London

Jacques Benveniste, the French immunologist who claimed that water has a 'memory' — a putative explanation for homeopathic medicine — died on 3 October in Paris after heart surgery. He was 69 years old.

Benveniste was a maverick whose theories on molecular signalling in cell biology were dismissed by many other researchers. Yet his charm, charisma and rhetorical flair earned him a large following in France, and he remained genuinely convinced that he had discovered something important.

Much of his celebrity sprang from a *Nature* paper in 1988 — published with an unusual rider from John Maddox, the journal's editor at that time, pointing out that, at face value, the findings were unbelievable (see *Nature* 333, 816–818; 1988). According to Benveniste's paper, water that had once contained biomolecules, but had been diluted until it was devoid of any active agents, could still have a biological effect. The paper described white blood cells involved in allergic responses being activated by such a solution of antibodies.

At the time, Benveniste was research director of the Clamart-based Unit 200 of INSERM, the French biomedical research agency, which studied the immunology of allergy and inflammation. He was temporarily suspended after Maddox launched an investigation of his methods with the help of fraud investigator Walter Stewart and stage magician James Randi. Benveniste complained that this amounted to a "Salem witch hunt".

Although Benveniste's results were widely disbelieved by scientists and were not reproduced elsewhere, they were hailed in the popular press as a validation of homeopathy. The 'memory of water' was never clearly explained; Benveniste suggested that quantum-mechanical effects might be involved.

Benveniste continued to investigate biomolecular behaviour at high dilution, and concluded that molecular signalling occurs by electromagnetic transmission. He published claims that he had induced cell responses by playing back digitally recorded molecular signals in the absence of the molecules.

Although he kept up an association with INSERM, his studies during the past decade were conducted primarily at his privately funded Digital Biology Laboratory in Clamart.



Peace prize: Wangari Maathai is rewarded for grass-roots environmental work in Africa.

Kenyan vet garners Nobel for founding Green Belt Movement

London This year's Nobel Peace Prize was awarded to a former veterinary academic who moved on from university life to found a pan-African environmental movement.

Wangari Maathai, a former chairwoman of the Department of Veterinary Anatomy at the University of Nairobi in Kenya, was rewarded for establishing the Green Belt Movement. The organization, which has overseen the planting of tens of millions of trees across Africa, works with poor women and promotes local democracy

and sustainable development alongside its environmental programmes.

Maathai, currently Kenya's assistant environment minister, became one of the first women from central or eastern Africa to earn a doctorate when she was granted a PhD by the University of Nairobi in 1971.

Not all of Maathai's views endear her to researchers, however. This August she caused a media furore by expressing her view that HIV was bioengineered in the lab, rather than being a natural virus.

Salty diet provides square meal for shapely bacteria

Sydney Two groups of researchers have independently figured out how to grow square bacteria in the lab.

The microbe, called 'Walsby's square archaeon' after the researcher who scooped it from a pool near the Red Sea in 1980, is unusual both for its love of salt and its shape. "They look like postage stamps," says Henk Bolhuis of the University of Groningen in the Netherlands, who led one of the groups. Bolhuis's team cultured the slow-growing bacteria in a solution about as salty as soy sauce (H. Bolhuis *et al. Environ. Microbiol.* doi:10.1111/j1462-2920.2004.00692.x; 2004).

"It's an exciting breakthrough for studying the ecosystem," says Mike Dyall-Smith,

whose team at the University of Melbourne, Australia, also cracked the problem with a similar technique (D. G. Burns *et al. FEMS Microbiol. Lett.* 238, 469–473; 2004). Scientists think that similar 'extremophiles' might be able to survive in the salt-rich brines of Jupiter's moons Europa and Ganymede.

Gene variation centre is a snip at \$14 million

Washington A genotyping centre has been set up at the Massachusetts Institute of Technology to help researchers hunt for genes that cause disease.

The Eli and Edythe L. Broad Institute has received \$14 million from the National Institutes of Health to perform large-scale screens for single nucleotide polymorphisms, or SNPs (pronounced 'snips'), in individual genomes. SNPs are spots in the genome that vary extensively from person to person, and make up the small percentage of the genome that accounts for our genetic individuality.

Stacey Gabriel, who will head the centre, says she hopes to screen genomes for up to a million SNPs. The methodology will be completely open, says Gabriel, with the intent of helping other centres to analyse SNPs too: "We don't just want to hand out genotypes."

Maurice Wilkins, DNA's third man, dies at 87

London Maurice Wilkins, who shared a Nobel prize for the discovery of the structure of DNA, died in hospital on 6 October at the age of 87. Wilkins was still a staff member at King's College London, where he had worked since 1946.

Wilkins' fellow 1962 Nobel prizewinners, James Watson and Francis Crick, received more plaudits than Wilkins for their realization that the DNA molecule twists into a double helix. But Wilkins' research on a pioneering technique called X-ray fibre diffraction provided the proof that Watson and Crick needed to back up their theory (M. H. F. Wilkins, A. R. Stokes and H. R. Wilson *Nature* 171, 738–740; 1953). Until then, X-ray images could only be derived from crystals. Wilkins worked on the DNA project with Rosalind Franklin, who died before the Nobel prize was awarded.

Wilkins also worked in the Second World War on the Manhattan Project to build an atomic bomb, improving the process that isolates radioactive uranium. Later in life he was heavily involved in the Campaign for Nuclear Disarmament, and toured the world to promote social responsibility in science.

A full obituary will appear in next week's issue.

Dolphin nets protection at trade conference

Tokyo Moves were made last week to protect a rare dolphin, a plant with pharmaceutical properties, and caviar from the endangered beluga sturgeon. Trade in all three was restricted at the conference of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Bangkok, Thailand.

A decision was taken to ban all sales of the Irrawaddy dolphin (pictured), of which there are thought to be only about 1,000 left in the wild. There is an active market for these endangered dolphins in aquatic shows, and many are accidentally killed in fishing nets.

Trade of the hoodia plant, used by southern Africa's natives to suppress appetite and by drug companies in anti-obesity pills, will also



be restricted by CITES. This should help to boost sustainable development of the plant, representatives said.

At the same meeting, the Caspian Sea states of Azerbaijan, Iran, Kazakhstan, Turkmenistan and Russia agreed to cut their total quota for exported beluga caviar by 50%.

Cow sheds secrets as genome is unveiled

London The cow has joined the growing farmyard of animals whose genetic sequence has been determined. An international consortium of researchers deposited a draft of the bovine genome from a Hereford cow in genetic databases on 6 October.

Researchers say the sequence will aid comparisons between the DNA of humans

and other mammalian species, and help to determine which genetic sequences are important in human biology and disease.

It should also help scientists who study agricultural diseases such as mad cow disease or foot-and-mouth disease, with a view to improving animal health and food safety. The plan is to sequence additional breeds of cow, such as Holstein, Angus and Jersey, in a bid to uncover genes involved in meat and milk production.

Cold comfort

The next generation of Antarctic research stations is now being designed and built. Quirin Schiermeier reveals the problems that architects, engineers and inhabitants must overcome in the Pole's unforgiving conditions.

The wailing sound of a saxophone can sometimes be heard echoing through the night on Dronning Maud Land in Antarctica. Anna Müller, a physician and the base commander of Germany's Neumayer research station, plays the sax to banish the loneliness and boredom that creep up during the dark, freezing polar winters.

Life in the narrow station has left its mark on the four men and five women who have wintered on the 12-year-old base, now buried under 20 metres of snow. During the long winter months there was little scientific work to do here, other than the daily checking of meteorological instruments. Spring has finally arrived, but during July and August some crew members did not leave the window-less tubes for weeks. Only in December, when the summer crew arrives, will life in this voluntary prison become more enjoyable again.

Neumayer is just one of about 100 permanent research stations that have been built in Antarctica since 1904, when the Scottish explorer William Speirs Bruce built a year-round meteorological observatory on the South Orkney Islands, at 60° south. Now several countries, including Germany, Britain, the United States and France, are busy building or planning the next generation of stations. These will offer tomorrow's polar researchers more comfortable living conditions, but they will also have to meet tighter environmental regulations.

There are currently 82 stations, maintained by 27 countries. About half of them are permanently occupied. These scattered outposts at the end of the world (see map) provide bases for a wide range of research,

from astronomy and climate sciences, to geology and marine biology. They are pretty much the only man-made structures in Antarctica — the Antarctic Treaty, first signed in 1959, ensures that the continent is used only for peaceful, non-military purposes, effectively reserving it for adventure, a little tourism, and a lot of science.

Many of the science bases on Antarctica are long overdue for rebuilding. Some are slipping off the edge of the continent. Others are buried by metres of ice, thanks to yearly snowfall. Only the tip of the 30-year-old US South Pole Station now pokes above the surface, forcing researchers to enter via a steep staircase in a snow well. Some stations are simply bare-boned shacks that leave

researchers prone to depression during the winter months.

Although a few of these ancient huts are being preserved for posterity — such as a British station built in 1944 on the island of Port Lockroy — most are being revamped. At the Pole, for example, the sinking Amundsen-Scott South Pole Station is set to be replaced by a US\$150-million complex, scheduled for completion next year.

Special delivery

Building at such sites is a mammoth logistical challenge and the new complex has taken almost 15 years to plan and construct. The Pole is about 3,000 metres above sea level, and the thousands of tonnes of material for the base and nearby research facilities — including IceCube, a new high-energy neutrino observatory — had to be delivered by cargo planes or bulldozer convoys battling their way over ice and crevasses from the US coastal base at McMurdo Sound more than 1,600 kilometres away.

The new station will be separated from the outside world by thick steel doors and airlocks. Before polar air enters its heating and air-conditioning systems, it will be raised to room temperature by passing it through a heated glycol solution. And should something go wrong, most of the station's life-sustaining systems — heating, electricity and medical facilities — are designed to be remotely operated and repaired, via a computer link, from institutes in the United States.

Ironically, these provisions for a frozen world were designed by the Hawaiian-based architect Joe Ferraro, who can see the





Halley V (left) risks being washed away, and the South Pole Station (middle, right) is drowning in ice.

palm-tree-lined beach of Honolulu from his office window.

About 1,600 kilometres north — although every direction is north if you start from the South Pole — a smaller wintering station is currently under construction at Dome C, a high plateau ideal for ice-core drilling (see *Nature* **429**, 596–597; 2004). Concordia, built jointly by France and Italy, consists of two octagonal buildings in which a 16-strong crew will conduct year-round experiments on everything from geomagnetism to cosmic background radiation, starting next year.

To beat the encroaching ice, Concordia's buildings rest on legs that can hydraulically lift the entire station, keeping it metres above the snow from year to year. Several stations already use such systems, including Britain's Halley V Research Station, and they look set to be a standard feature in the polar architecture of the future. The hydraulic legs prevent the accumulation of snowdrifts and allow for windows, although they also expose the crew to the loud noise and strong vibrations caused by polar storms.

Mobile homes

The Halley base faces an even tougher challenge than Concordia and the US South Pole Station: it sits on a coastal shelf ice that is moving at 100 times the speed of inland glaciers. Building on such mobile foundations certainly puts an architect's knowledge of ice and snow to the test. As snow accumulates inland, the ice flows towards the ocean at several hundred metres per year, eventually breaking off in icebergs, so each structure has a limited lifetime of no more than 20 years.

Britain and Germany are the only two countries maintaining such shelf stations, and both are currently designing new bases

in northern Antarctica: Halley VI on the Brunt Ice Shelf and Neumayer III on the Ekström Ice Shelf. The British Antarctic Survey warned in April that Halley V could be washed into the Weddell Sea the next time a massive ice sheet breaks off the shelf, which is due to happen around 2010. And at Neumayer II, snow pressure is already causing scary distortions and deformations of the existing tube-shaped building.

The new stations will be built on carefully selected sites to ensure the longest possible life before the ice slips into the sea. Both will be jacked up on extendable hydraulic stilts. And the German buildings will be enclosed in a large outer structure, creating a buffer zone between the living space and the outer skin for extra insulation and protection from storms.

But aside from these few requirements, the final designs for these buildings have yet to be finalized. The design for the UK station will be selected through a competition in September 2005, with entrants' proposals expected to encompass some of the most innovative — and most cost-effective — ways to ensure a happy, healthy crew.

To comply with strict environmental regulations introduced in 1998, the buildings will also have to restrict their use of water and fuel. Waste heat from diesel generators will be harnessed for extra warmth, and wind and solar power will be high on the list of desirable technologies for the new stations. At the moment, a station the size of those planned by Britain and Germany consumes some 200,000 litres of diesel per year.

Technology developed by the European Space Agency (ESA) could help, says Fritz Gampe, a senior officer in the agency's technology-transfer programme. An automated water-treatment plant developed for use on the International Space Station, for

example, could easily be installed in polar stations, he says.

And a prototype 'space house' that the agency developed for use in earthquake-prone regions or a possible future Mars colony would fit polar requirements perfectly, he adds. The solar-energy-powered structure, which looks like a futuristic igloo built of ultralight carbon composites, would also be easy to transport home once its lifetime on the ice had expired.

ESA has pitched just such a proposal for the Neumayer station. But Dietrich Enss, a consultant engineer with the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, which maintains Germany's Antarctic bases, is sceptical. "What we really need in Antarctica is maximum success at minimum cost," says Enss. "After all, this is not a beauty contest down here, but all about science."

Nonetheless, beauty and comfort are important on the insides of these structures. "Communal space is key for the team spirit in winter, so improving the human aspect of accommodation will be an important criterion," says Malcolm Reading of the London-based architecture firm Malcolm Reading & Associates, which is running the design competition for the British station. Viewing domes and adjustable interior walls for creating larger, temporary communal spaces, would be welcome features, he says.

Feathered friends

Reading recently asked staff who winter at British Antarctic bases what they miss most during their stay. "Pets" and "the laughter of children" were the most frequent answers. Although no children have yet been spotted in Antarctica, pets have. Tourists cruising the shoreline of the continent in summer occasionally report seeing budgies in cages outside the scattered stations, despite the fact that this is technically a violation of the Antarctic Treaty, which bans the import of all non-indigenous species to the continent in order to protect its fauna and flora.

Pets and new-fangled architecture aren't looked upon kindly by some of the veteran explorers of the continent. "A little bit of hardship is good for you," says Peter Clarkson, a geologist and executive secretary of the international Scientific Committee on Antarctic Research, who was base commander of Britain's Halley II station in 1968. "If you're sitting there inside a centrally heated building in T-shirt and sandals things just aren't right."

But for most of the younger scientists who live in Antarctica over the winter, including Müller, a new generation of stations complete with windows would be an immense relief from the winter blues. "My stay here was a totally unique experience," says Müller. "But I wouldn't do it again." ■

Quirin Schiermeier is *Nature's* German correspondent.

Science in the FAST LANE

With the rules of the game changing before every season, Formula 1 engineers often have a matter of weeks to redesign their car before it is tested on the track. Karl Ziemelis and Charles Wenz join the race to the start line.

Christine Lear's secret is out. As a practical girl, she chose a practical career path, one that suited well her university training in mathematics: she went into computers. Yet something didn't feel right. From an early age Lear has been fascinated by cars. And not just any old cars. Lear's once-secret obsession is with Formula 1, the pinnacle of high-octane motor racing.

"Working in Formula 1 has always been my dream," she now admits. But even when she got a chance to fulfil that dream — a research position in a university aerodynamics group with close ties to the racing-car industry — she kept her secret to herself. Convinced that it was just wishful thinking, she did not dare confess her hopes, and pursued a PhD investigating ways to reduce the hazardous airflow patterns that form in an aircraft's wake. Or, as she puts it, "how to land more planes at Heathrow".

But she never lost the excitement she felt during Formula 1 races whenever the commentator referred to 'new aero packages' and other tantalizing revolutions in car performance. She had always wanted to know what lay behind the jargon, and rather than regret never having tried, she finally decided to pursue her childhood ambition.

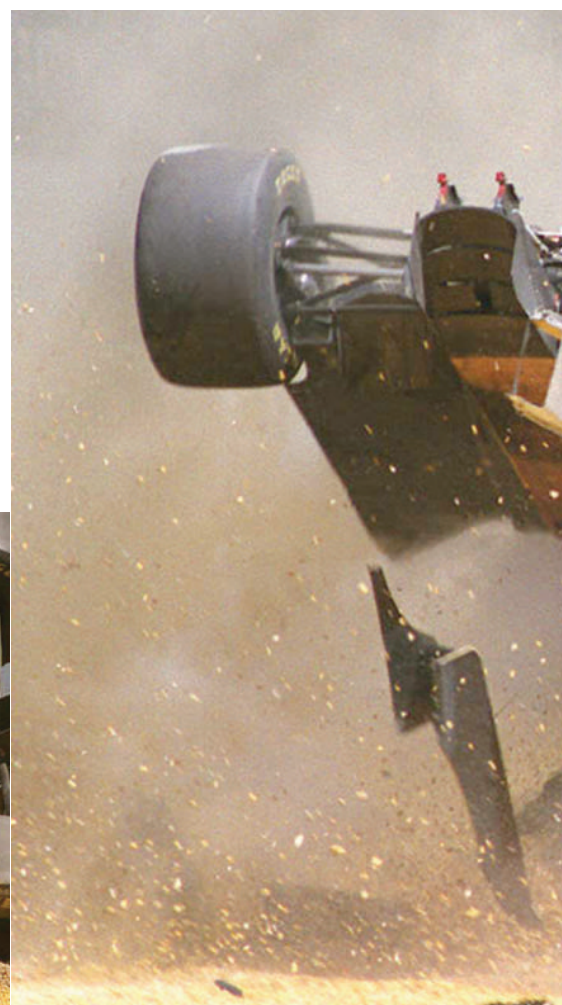
Lear now has the job of her dreams. In May she joined the Switzerland-based Sauber-Petronas team, with prime responsibility for developing key aerodynamic components on its Formula 1 car. "There was a running joke in the office about my very first job with the team," she recalls. "Take a woman and give her the mirrors." But having proved her worth, she now has a new secret to keep — in this highly competitive industry she is not allowed to divulge the details of projects she is working on.

Academic research has its highs and lows, but there is always some comfort in knowing that the laws of nature underlying your work are likely to be fixed, just waiting to be revealed. If it can be considered a race at all, then it is simply a race to be the first to uncover these inner workings.

The pressures of research in Formula 1



Crash course: engineering teams have significantly improved safety in Formula 1 — even during accidents — and with data from cars on the racetrack (right), they have also boosted performance.



are very different. The laws of nature may be unchangeable, but not so the laws of the sport. The rules under which the teams operate alter from season to season, motivated by a desire to keep car speeds within safe limits while making the cars unpredictable enough to keep drivers — and spectators — on their toes. The performance of a car that is hard to handle at speed ultimately depends on the skills of the person behind the wheel, which is why Formula 1 has champions, not mere drivers.

Rapid reactions

The car designers, of course, have a very different goal. For them, their machine needs to go as fast as is physically possible. It needs to handle like a dream, glued to the road in the tightest of corners. And it needs to win.

Whereas an academic may view the idea of abandoning a cherished line of enquiry with considerable dismay, the same is not true for race engineers. "Rule changes are never a setback," says Peter Bearman, director of aerodynamics at Imperial College London and mentor to Lear and numerous other Formula 1 recruits. "They relish the challenge."

Willem Toet, senior aerodynamicist with

the BAR-Honda Formula 1 team, agrees. Back in the spring of 1994, car performance had reached an all-time high and, over one race weekend, tragically culminated in the deaths of two Formula 1 drivers — Austrian newcomer Roland Ratzenberger and Brazilian champion Ayrton Senna. In their bid to improve car safety, the rule-makers imposed drastic changes to the design specifications, giving the teams only two weeks to comply. By restricting the dimensions of critical aerodynamic components, their aim was to reduce the 'downforce' (and hence speed) that the cars could sustain under race conditions.

Toet remembers the ensuing flurry of activity well. He and his colleagues resorted to cutting away chunks of the finely honed bodywork of their car to crudely bring it into line with the new specifications. The downforce of the butchered machine immediately dropped by 30%. But within a matter of days, redesign and tweaking of the downsized components helped them to recover half of the lost performance, maintaining the car's competitive edge. "Extreme physical and mental work was required — a true intelligence test," says Toet.

This high-pressure environment may



help to explain why Formula 1 recruits heavily from academia. “My last three PhD students all went into Formula 1,” says Bearman, whose department has now provided about 40 engineers for the industry. One of his current students, Jonathan Pegrum, is clear about what attracts him to Formula 1. “I want to use what I have learned,” says Pegrum, “in aerodynamics, it is hard to think of another industry that is at the cutting edge.”

“Many of the people we employ on the engineering side come straight from university,” says Nicholas Tombazis, chief aerodynamics engineer at the McLaren-Mercedes

team. Tombazis especially values the solid understanding in aerodynamics that academic training provides, and his team currently supports two PhD projects in Bearman’s group.

But this has not always been the case. Although there is now genuine admiration in the engineering community for the technical achievements that underpin a Formula 1 car, in the early days of the sport it was viewed with more suspicion. “Cowboys playing with fire,” according to Brian O’Rourke, chief composites engineer at the Williams-BMW team.

Rewind to the early 1970s. Recognizing its lack of aerodynamic expertise, the Formula 1 industry approached the aeronautics group at Imperial College to help solve a nagging problem: cooling. The cars of that era had a front-mounted radiator, the air from which was funnelled up through the nose of the car and — inconveniently — out through the cockpit. The result? One driver, medium rare.

Solving this problem resulted in a fundamental rethink about the positions of the radiators themselves (they are now side-mounted), followed by a redesign of the car’s nose into the now-familiar, and more aerodynamically efficient, pointed shape.

Taming the force

This was also a time when wind-tunnel techniques underwent rapid development — most notably the introduction of ‘rolling roads’. The flow of air around a moving car depends not only on its shape, but also on interactions with the road underneath. To correctly mimic a car moving at speed, the underlying surface needs to move in pace with the flowing air, rather than staying motionless as was previously the case.

A deeper understanding of the interactions between car, air and road led to new ways of exploiting aerodynamic effects. Instead of focusing mostly on drag reduction, engineers discovered that you could make tremendous gains in performance by using aerodynamics to stick the car to the road. This downforce is essentially the reverse of the ‘lift’ that keeps planes in the air, and is the main role of the ‘wings’ attached to the nose and rear of the car.

In the 1970s, Lotus engineers realized that the entire car could be made to act like a giant inverted wing if the underside was suitably contoured. This concept became known as the ‘ground effect’, and led to the introduction of various devices — including road-hugging skirts — to exploit it. Most were then predictably banned. Despite all the rule changes, the combined downforce and ground effect on modern cars would be sufficient, in theory, for them to drive upside down on a ceiling at high speed.

Plane sailing

But even with these aerodynamic principles firmly in place, designing cars remains a challenge — when it comes to controlling airflow around the chassis, racing cars have a decidedly nasty shape. And the cars have different aerodynamic needs depending on the track conditions — meaning specific features change from race to race. So new aerodynamic ideas will continue to feature heavily in Formula 1 racing, and the teams all now have highly qualified aerodynamicists devoted to the task.

It has been said that a modern Formula 1 car has more in common with a jet fighter than an ordinary road car. But few would

think of racing car aerodynamics as being more complex still. According to Xin Zhang, an aerodynamicist at the University of Southampton, UK, Formula 1 has pushed the science and engineering expertise in some areas “to a level higher than that of aerospace engineering”. In his view, understanding the flow around the streamlined body of an aircraft is straightforward by comparison.

And despite the secretive nature of the Formula 1 industry, the flow of information from aeronautics to racing is beginning to reverse. When Britain’s Ministry of Defence began developing the current Harrier fighter jet, the GR.7, it drew on the expertise in composite materials accumulated by Formula 1 engineers.

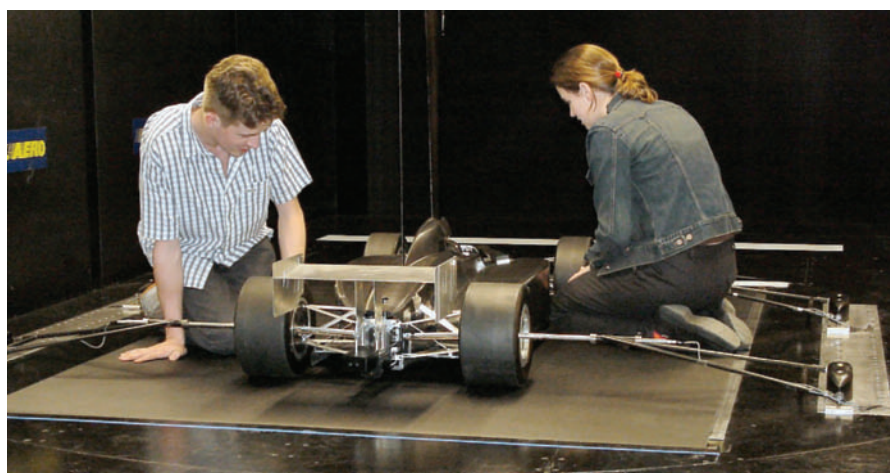
In Formula 1, everyone likes an accident that people walk away from. Certainly, the race coverage revels in spectacular high-speed accidents from which the drivers emerge relatively unscathed before offering a cheery, yet relieved, wave to the watching fans.

Of course accidents don’t happen by design. “We’d far rather the drivers finish the race safely,” says Steve Foster, head of composite design at the Jaguar-Cosworth team. But combining a thoroughbred race car with the drivers’ passion for victory results in a heady mix that occasionally ends in catastrophe. Formula 1 drivers are well aware of the risks involved when taking to the track. And behind the scenes, materials scientists such as Foster and O’Rourke are working hard to improve driver safety.

When composite materials made their debut in Formula 1 in the early 1980s, they were little known to the racing industry, and even less understood. As O’Rourke recalls, the teams were keen to exploit materials with high stiffness and low weight — advantages that they had heard about but did not know how to achieve.

Later, when compulsory crash testing of the driver cockpit was introduced in 1988, attention shifted away from stiffness to the more complex material property known as ‘strength performance’. This is essential for ensuring driver safety during impact. Today, the composites that make up the cockpit have to survive multiple impact tests before the car is even allowed to race. “Modern composites enable levels of crashworthiness and protection that would be impossible to achieve with traditional materials,” says Foster.

Despite these successes, working with these materials is too often a black art, and this is where the team has its work cut out.



It’s a blast: aerodynamics students assess a racing prototype in a wind tunnel at Imperial College.



Living the dream: Christine Lear has fulfilled her racing ambition.

“There is great breadth and depth of knowledge in the research community,” says O’Rourke, “but the chances of anyone looking at the exact configuration we need are small.” Still, he adds: “It is very useful — not to say comforting — to know who the main names are when you get into a difficult situation and need to ask an opinion.”

Tuned in

And when time and money allow, a Formula 1 team will commission a piece of research work, ranging from an undergraduate project right up to supporting a PhD thesis. “I have been involved in around 20 such studies over the years,” says O’Rourke, although he notes that the projects have to be chosen carefully to balance the teams’ and the students’ needs.

Formula 1 has much more glamour than academia, but is it the best career move for a young researcher? After all, they are unlikely to have as much control over their research as in academia, the hours are long but they won’t publish anything, and they will experience little of the Monte-Carlo lifestyle of the celebrity drivers. Even attending a race is a rare event. So is it worth making the switch?

For Lear, much of the excitement comes

from the rapid returns. In contrast to the sedentary pace of university research, development of a Formula 1 car takes place at a breakneck speed. “What I am working on right now could appear on the car in a race or two,” she says. Barney Garrood, a recent recruit to the Ferrari team, says that this was a key factor in his decision to make the move from academia. “Research in the aerospace sector is static in comparison, with typical development times of 10 to 15 years,” he says. “This is much more exciting.”

Then there are the resources. The multi-million-dollar budgets behind a Formula 1 team would be the envy of most research groups. No surprise then that Bearman refers to the McLaren Technology Centre in Woking, UK, as “a temple to technology”. With its sweeping architecture and state-of-the-art facilities, this striking establishment was designed to inspire. “McLaren wanted to create an environment that will motivate and influence people who work within it,” says Tombazis.

For Lear and her former academic colleagues, gone are the frustrations of limited technical support, or killing time while waiting for a slot on a university’s over-subscribed facilities. These young guns now find themselves immersed in a continuous and rapid cycle of designing, building and testing. “The aim is to have new parts on the car for every race,” says Lear.

And like most researchers, those in Formula 1 get a kick from knowing their work makes a difference. The goals may be more limited in a strict scientific sense, but the work has more immediacy than in almost any other field of enquiry. “Being able to point to your own parts on a car — and then seeing that car win a race — is a real thrill,” says Garrood.

And for Lear, perhaps the biggest thrill of all comes from having realized her dream. In a secretive profession, this is one secret that she is more than happy to share.

Karl Ziemelis and Charles Wenz are *Nature’s* resident petrolheads.

Best scientific advice is to read the climate report

Most researchers agree on the need to back Kyoto: let politicians deal with the politics.

Sir — Your Editorial “Not just academic” (*Nature* 431, 1; 2004) on the political climate in Russia stresses the importance of respecting academic freedom. But this is beside the point. To avoid bowing to political pressure, or indeed political incentives, Russian scientists should simply stop giving any political advice (either unsolicited or solicited) and confine themselves to developing genuine scientific concepts that reflect reality, not opinions.

Most conscientious researchers can distinguish the ideological and scientific

aspects of the problems they study, and keep them separate. The existing range of scientifically justified concepts on climate change provides a wide, but well delineated, field for political manoeuvring. There is a broad consensus among climate scientists that humankind must urgently develop legal, institutional and financial mechanisms for regulating climate. The extent of regulation that's needed is open to debate, but most scientists support the Kyoto Protocol as the first practical step towards this goal.

However, the problem of assessing the costs of such actions is traditionally tackled by politicians, not scientists. If we all agree that such problems are best addressed through politics, it is clear that the only advice a conscientious researcher can give to a Russian politician is as follows: “Read the reports of the Intergovernmental Panel on Climate Change.”

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Climate: Russians face another disappointment

Sir — In the News Feature “Crunch time for Kyoto” (*Nature* 431, 12–13; 2004) you mention the lack of public pressure on Russian president Vladimir Putin to begin tackling climate change and ratify the Kyoto treaty. There seems to be a widespread belief in Russia that climate change is a peripheral issue, unrelated to the supply of jobs, to putting a roof over your family's head or food on your table. Add to this the belief that a warmer climate would be an improvement — taking some of the chill out of winter and maybe increasing harvests — and it's little wonder that climate change isn't on the lips of every Russian voter.

However, the reality of climate change for Russia, and boreal Asia as a whole, is unlikely to be the balmy-weathered, bumper-harvest future some expect. Although increasing temperatures may well allow extended growing seasons and a northward shift in crop zones, increased damage from pests, drought and severe weather could lead to a 30% cut in cereal production by 2050 across the region, with Siberia seeing a decrease of up to 20% in agricultural output (see Intergovernmental Panel on Climate Change *Climate Change 2001: Impacts, Adaptation and Vulnerability* Cambridge University Press, Cambridge, UK; 2001).

Even if the net effect is an increase in food production, it is likely to come at a price. Industries such as mining and construction face soaring costs as a result of melting permafrost, increased flooding and building subsidence. The health and transport sectors could come under huge additional pressure, and international tensions are likely to be inflamed by water shortages, famine and mass migration in other parts of Asia.

For our Russian voter then, living through Russia's already painful social and economic post-Soviet transition, climate change threatens to make life more painful still.

David S. Reay

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Passion and politics cloud the climate debate

Sir — I question the view expressed in “Crunch time for Kyoto” (*Nature* 431, 12–13; 2004) that Russian attitudes towards the Kyoto Protocol are “heavily influenced by its dented pride and need for respect”, as a former superpower. I suggest that concerns held by members of the Russian Academy of Sciences about Kyoto, and their surprise at British delegates' complaints about the inclusion of climate-change ‘sceptics’, are shaped by disquieting memories of the Soviet era.

It is 40 years since the end of Trofim Lysenko's dictatorship over Soviet biology. A poorly educated agronomist, Lysenko gained political support during the agricultural crisis of the 1930s by denouncing conventional genetics as a “bourgeois deception” and promising improved crop yields on the basis of crude and unsubstantiated “experiments”. He became the autocrat of Stalinist science, with catastrophic results that linger today.

Lysenkoism was a tragic example of an illusion that became accepted as reality, despite all contrary evidence, because it was continually affirmed at meetings and by the media — see V. N. Soyfer's *Lysenko and the Tragedy of Soviet Science* (tr. L. & R. Gruliov, Rutgers University Press, New Brunswick; 1994). In 1965, Academician

and Nobel prizewinner Nikolai N. Semyonov was finally able to write: “There is nothing more dangerous than blind passion in science. This is a direct path to unjustified self-confidence, to loss of self-criticalness, to scientific fanaticism, to false science. Given support from someone in power, it can lead to suppression of true science, and, since science is now a matter of state importance, to inflicting great injury on the country.”

Russian distrust of the interaction between science and politics remains strong. To many of the academicians, as to many of their colleagues around the world, the global-warming paradigm is far from ‘fact’, but objective debate is distorted by political and commercial interests. In this context, I suggest that it was perfectly reasonable for the academy's programme to include scientists with a range of viewpoints. It was unfortunate that the British delegation tried to exclude a selection of these because — as one member is reported as saying (*Science* 305, 319; 2004) — “We knew that we would not get to the scientific issues if we went down every rabbit hole of scepticism.”

The “sceptics” who were invited, including myself, were not speaking about the Kyoto Protocol. For my part, I argued against claims that malaria and other mosquito-borne diseases are spreading to new latitudes and altitudes because of climate change.

I feel no offence at being branded a sceptic — quite the contrary — but I never dreamt that I would hear a top Russian administrator at a Kremlin press conference refer to me, a British scientist, as a “dissident”, and to the representatives of the British government as “totalitarians” who had tried to “censor” me to protect their Party. Truly, an irony of history.

Paul Reiter

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The state of the Universe

A bold attempt to make sense of relativity, quantum theory and cosmology.

The Road to Reality: A Complete Guide to the Laws of the Universe

by Roger Penrose

Jonathan Cape: 2004. 1,094 pp. £30

Jeffrey Forshaw

"The most important and ambitious work of science for a generation." That's the claim from the publishers of Roger Penrose's latest book. The claim is vastly overblown. Certainly Penrose has written a remarkable book: it introduces many of the topics that lie at the cutting edge of research into the fundamental nature of space, time and matter. Although the book aims at a complete survey of modern particle physics and cosmology, its principal concern is to address the fundamental tension between the two pillars of twentieth-century physics: Einstein's general theory of relativity and quantum theory. This is a fascinating tension and one that Penrose tries to communicate in a quite uncompromising fashion.

Although advertised as popular science, this book will be far from accessible to most non-experts. I suspect that there has never been such a bold attempt to communicate ideas of such mathematical complexity to a general audience. It is Penrose's hope that non-experts will be able to go with the flow and get a taste of the excitement of the field without following the details. Unfortunately, the book is crammed full of details and is so utterly uncompromising that it will probably leave even the most enthusiastic of his non-expert readership exhausted. Even those with a PhD in mathematics or physics are likely to find it very hard going.

Having said that, the book is teeming with delights. Penrose's ability to present complex ideas in a logical, coherent manner, often using geometry, reflect a deep understanding that will seriously engage a more expert reader. There are even exercises, graded "very straightforward", "needs a bit of thought" and "not to be undertaken lightly", which the reader is encouraged to attempt. I certainly found the classification of this final category quite appropriate.

The opening chapter, on the roots of science and the notion of mathematical truth, marvels over the ability of mathematics to explain so much of the behaviour of the world with such incredible precision. Some scientists may balk at questions of ontology, preferring to banish them to the realm of metaphysics, but this is not Penrose's way. In his subsequent assessment of the measurement problem in quantum mechanics, he argues forcefully for the need for a credible ontology. The book ends with reflections on



Up the junction? Despite progress in many directions, we still haven't found the one "road to reality".

the future, critically assessing the way in which physicists progress in their quest for an ever deeper comprehension of reality, and offering some personal views on the sociology of contemporary research. In between there are 32 chapters of serious mathematical physics with little room for philosophical reflections.

So serious is Penrose in his desire to present the science in a worthy fashion that one has to wait patiently (or struggle valiantly) until chapter 17 for the physics. The mathematics of the first part of the book is a joy to read. Not all of it is needed for the later chapters, and the selection and presentation of material clearly reflects Penrose's love of the mathematical structures that underpin the physics.

Penrose starts from a clean slate, encouraging the reader to question the dominant role of real numbers, or the validity of euclidean geometry, in describing the world around us. Much time is devoted to complex numbers and their "magic". In typical fashion, Penrose does not shy from introducing Riemann surfaces, holomorphicity and hyperfunctions. Hot on the heels of these come quaternions, leading naturally to a discussion of Clifford and Grassman algebras. Chapters on differential geometry and symmetry conclude the presentation

of the necessary mathematics. The section ends with a chapter — not strictly relevant but entirely captivating — on the notion of infinity, covering Cantor's work on cardinal numbers and Gödel's incompleteness theorem.

Then the physics starts, with a chapter on space-time. The earlier chapter on differential geometry has paved the way and the reader is ready to understand Galileo's principle of relativity as implying that "space is a bundle over time". Einstein's idea that space and time might constitute a unified whole is by now an entirely reasonable a priori possibility.

The following chapters introduce field theory and quantum theory. Much of the discussion on the measurement problem in quantum theory will be familiar to those who have read Penrose's earlier book *The Emperor's New Mind*. In *The Road to Reality* he lays out his contention that quantum theory, as currently formulated, must be incomplete, and this is a recurrent theme for the remainder of the book.

There is a rather cursory chapter on the standard model of particle physics, which rather confusingly precedes the chapter on quantum field theory and makes the error of stating that weak interactions couple only to left-handed particles. It is also stated that the only measured violation of charge

parity, a symmetry that ultimately helps to account for the preponderance of matter over antimatter in the Universe, is in the study of neutral kaon mesons, a declaration that will not be popular with those working on certain experiments based in the United States and Japan.

The main thrust of the final part of the book is the exploration of physics in those often exotic places where both quantum mechanics and gravity play a role, such as at the birth of the Universe or in the vicinity of a black hole. The very existence of the second law of thermodynamics is used to argue that the Universe must have started out in an extraordinarily special configuration (something else that will be familiar to readers of *The Emperor's New Mind*), which Penrose takes as a hint that the quantum theory requires revision.

After a more detailed discussion of the measurement paradox, Penrose is ready to expound his belief that quantum state reduction is an objective process, arising as a consequence of the gravitational energy difference between the different space-time geometries possessed by quantum states in superposition. By now, Penrose is becoming increasingly partisan, although he is careful to announce when he is deviating from the "accepted wisdom". He is forthright in stating his objections to several mainstream ideas: inflationary cosmology, low-energy supersymmetry, the electroweak phase transition and string theory are all targets. Indeed, his presentation of string theory is essentially a technical critique, and I suspect it will be inaccessible to non-experts. At times his objections can lead to rather judgemental and acerbic statements: the leading string theorist, Ed Witten, is described at one point as a "tour guide".

Having been so critical, Penrose offers his thinking on the possible way forward for a consistent theory of quantum gravity. Unfortunately these two chapters are the most challenging of the whole book. Loop variables, spin networks and his own twistor theory are all presented, although he acknowledges that these ideas have not yet come close to matching up as a viable physical theory.

In sum, this book certainly doesn't live up to the publisher's hype. It is too technical to be accessible to a general audience and is focused on only one branch of modern science. However, it is carefully crafted and rich in deep insight. Penrose's own hand-drawn diagrams help to remind the reader of the very personal account provided in its pages. Penrose, leading by example, clearly intends that this book should encourage scientists to dare to be original in their quest: in that, he may well have succeeded. ■

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Field guides and phylogenies

Flowers of Ethiopia and Eritrea: Aloes and Other Lilies

by Sebsebe Demissew, Inger Nordal & Odd E. Stabbetorp
Shama Books, Addis Ababa, Ethiopia: 2004.
227 pp. US\$15
e-mail: shamabooks@telecom.net.et

Sandra Knapp

Taxonomy has been much in the news in the past year or so. Everybody seems to want more of it, and in a slightly different way than it is currently done. Perhaps taxonomy should be done solely on the web, or by using a set of DNA barcodes — opinions differ. But just what is taxonomy and why is it suddenly in demand? I contend that taxonomy (or systematics, as some people prefer to call it) is composed of three interlocking spheres of scientific endeavour: phylogeny, description and identification. This book is a sterling example of how all three aspects of taxonomy can come together to produce something of lasting value to a variety of end-users.

The book is a colour guide to the identification of the charismatic flowers that characterize dry habitats such as Ethiopia — 'lilies' in the broadest sense. Identification is aided by easy-to-use keys, simple descriptions and lovely photographic plates, which make this a nice book for just exploring the amazing diversity of these plants, quite apart from its obvious use in the field. But the authors have done more than just produce an identification guide: they have set the lilies of the region in their phylogenetic context,

and the book's introduction shows just how important it is to link those three areas of taxonomic study.

The phylogeny of the monocots (such as lilies, grasses and orchids) has undergone radical change and restructuring with the use of DNA sequence data. The number of families recognized and their relationships to one another have changed considerably over the past decade (see *Bot. J. Linn. Soc.* **141**, 399–436; 2003). Rather than brushing all this under the carpet, the authors clearly and concisely explain why phylogeny matters. Then, through the use of the field-guide format, they show how such rearrangements make sense when looking at the plants themselves. They are also brave enough to admit what all taxonomists know: that the classification of such groups, where new data are emerging, is still in flux.

Phylogenies and field guides are both of obvious use, but both require a solid base of descriptive taxonomy. This book, and to a certain extent the phylogeny to which it adheres, rests on the descriptive work done on the region's flora. Major projects documenting national floras (and faunas) have uncovered new species and provided the observations and taxonomic decisions that are needed for both phylogeny and field identification.

Books such as this, with its numerous illustrations and accessible style, inspire others to study organisms, helping the accumulation of information about uses and, to my mind more importantly, conservation status. How on earth can we conserve biodiversity unless we know something about it? This book is an example of how to do it. ■

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Sculpture

Opening time

On a summer evening several years ago, Eduardo Catalano, emeritus professor of architecture at the Massachusetts Institute of Technology, watched a flower as it was starting to close its petals. Suddenly, he realized what the movable structure he had so long wanted to create should look like.

That moment of inspiration resulted in 13 months of construction work. The outcome was *Floralis Genérica*, a stunning, gigantic flower (right) made of an aluminium alloy covered in stainless steel, intended to represent all flowers. It is 20 metres high and weighs 18 tons. Each of its six petals is 13 metres long and 7 metres wide, and they are mounted on a conical structure. A computer-controlled hydraulic mechanism opens the flower at sunrise and closes it at dusk. Red light is projected on the inner surface of the petals when the flower closes.

Born in Argentina, Catalano donated the



flower to the city of Buenos Aires, where it was inaugurated in April 2002. *Floralis Genérica* has found its home in a large pond in the heart of the city. The flower is kept open round the clock on the first day of spring and on some public holidays. And whenever there is a new Moon — such as today.

Juliane Mössinger

F. BARBAGALLO

Modern museums

Creating Connections: Museums and the Public Understanding of Research

edited by David Chittenden, Graham Farmelo & Bruce V. Lewenstein
AltaMira: 2004. 379 pp. \$80, £61, €104.64 (hbk); \$34.95, £26.95, €46.23 (pbk)

David A. Micklos

In the nineteenth century, science museums evolved from being aristocrats' private 'cabinets of curiosities' to public vehicles for the exposition of science. They continued to develop during the twentieth century alongside the logical positivist view of science, which holds that the experimental world is connected to the real world through a complex set of rules. Because of this direct correspondence, experiments could, in effect, uncover truth. So science museums presented the public with the validated artefacts of science, and public education systems fed their students received facts that could, in theory, be learned by rote memorization.

During the second half of the twentieth century, the logical positivist conception gave way to down-to-earth 'use' theories, which emphasize the creative and problem-solving aspects of science. This led, especially in the United States, to increased emphasis on hands-on experience with the process of science. A new breed of science centre was developed, focusing on demonstrations of scientific principles and manipulative models. By the end of the century, these centres faced competition from music videos and virtual reality. Despite their best attempts to make science more appealing, science museums and centres have suffered declining attendance, and polls show only modest gains in the public's knowledge of science. Science museums and centres are responding by experimenting with ways to connect the public to scientific endeavour.

The set of essays in *Creating Connections* focuses on the movement dubbed 'public understanding of research' (PUR). One can discern three themes that shaped discussions at a 2002 conference of museum and media people from which this book emanated: an approach based on current, unfinished 'science in the making'; public involvement and dialogue; and the process through which scientists make sense of the world.

The two major public science museums in London illustrate polar reactions to the PUR mandate. Graham Farmelo explains that the Science Museum has grafted new appendages for public engagement to its admittedly stodgy collections. Its Wellcome Wing uses state-of-the-art computer and graphical presentations, and the Dana Centre is a multimedia-wired pub designed to draw young adults into science discussion.



Breaking news: Boston's Current Science & Technology Center interprets stories as they happen.

On the other hand, the Natural History Museum has turned itself inside out with its Darwin Centre, creating a public interface with the guts of its research collection, as Neil Chalmers explains.

The drier parts of natural-history museums worked in their time (and sometimes still do), because in wandering their halls one can feel connected to the explorer who happened upon the strange and wonderful items on display. By comparison, most science centres today are disconnected from the science they attempt to explain. The hands-on manipulatives that are their *raison d'être* are often too abstracted to provide any sense of personal connection. As Christine Cansfield-Smith explains, the Discovery Science Centre attempts to foster closer interactions with scientists by locating itself on a research campus of Australia's Commonwealth Scientific and Industrial Research Organization. The US National Science Foundation is also attempting to forge direct connections by providing educational outreach funds as a condition of major research projects.

A highlight of the book is Carol Lynn Alpert's engaging description of how the Museum of Science in Boston developed its Current Science & Technology Center. One cannot fail to be impressed by her story of how seven full-time staff have laboured to weave mini-exhibits, theatre, interviews with scientists, and Internet multimedia into their daily audience discussions of breaking news.

Allied to this news emphasis are 'science cafés' and other devices to engage the public in dialogue about the impact of science on society. Unfortunately, I fear that the desire for public conciliation has become so rampant in Europe that it has turned many otherwise gifted science communicators into apologists for science. Opinion polls show that although the public in the United States and Europe hold scientists in almost equal esteem, Europeans are much more distrustful of the scientific enterprise.

Favouring rhetoric over process might have serious consequences. For example,

genetically modified (GM) foods are widely banned in Europe, where, doubtless, few people understand the process through which a GM plant is produced. In contrast, hands-on experiments with gene transfer in bacteria have been common in US high schools since the mid-1980s, presumably providing students with some real insight into the GM debate. Despite nearly two decades of safe practice in the United States, simple gene-transfer experiments are still absent from most European schools.

A growing emphasis on standardized testing threatens to erase such gains in hands-on instruction, however, and push US science education back towards the realm of received facts. Although the US National Science Education Standards include process, historical and societal elements, these take a back seat to more easily measured content. Experiments are, after all, never a time-efficient means of memorizing scientific facts.

Sadly, this volume is largely mute on the issues and opportunities raised by standardized testing, despite the fact that school students are a large constituency of every science museum or centre. There is a market for the type of high-quality experiments that schools lack the time or facilities to conduct, although few science museums have invested in teaching labs. At my own institution, we have found that teaching labs can easily generate enough income to pay for the faculty and supplies needed to run them.

I agree with Rick Bonney and Don Pohlman, who say in the book that science museums can best aid formal science education by providing insight into the process of science, which may be missed in fact-driven classrooms. Perhaps it is time for science museums and centres to focus on providing semi-structured environments in which students can connect with the experiments that really drive science. ■

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Before the word

Language evolution: evolutionary vestiges may provide clues to the ultimate origins of human language.

Gary F. Marcus

If, as François Jacob famously argued, evolution is like a tinkerer who builds something new by using whatever is close at hand, then from what is the human capacity for language made?

Most accounts of the evolution of language have focused on characterizing changes that are internal to the language system. Were the earliest forms of language spoken or (like sign language) gestured? Did language arise suddenly? Or did it emerge gradually, progressing step by step from a simple one-word 'protolanguage' (limited to brief comments about the 'here and now') into a more complex system that combined individual words into structured meaningful sentences encompassing the future, the past and the possible — as well as the concrete present? Regardless of how these questions are resolved, if we seek the ultimate origins of language, we also need to look further back, beyond the first protolinguistic systems, to whatever prelinguistic systems may have preceded any form of language.

Possible prelinguistic precursors might include systems for planning or sequencing complex events, categorization, automating repetitive actions and representing space and time. In each case, there are parallels between candidate prelinguistic cognitive (or motor) precursors and systems found in language. For example, many animals are able to construct mental maps for navigation, and all known languages draw heavily on spatial metaphors. Thus, it is tempting to conclude that machinery for the mental representation of space plays some role in — or is at the very least available to — the machinery for language.

But parallels alone are not enough to establish shared lineage between two systems — they could instead represent convergent (independent) evolution. For example, a language system could have evolved its own machinery for automating repeated tasks, independent of pre-existing machinery for automatizing other cognitive functions.

A more telling way of establishing prelinguistic ancestry could come from evolutionary contrivances — properties of language that existed not because of some selective advantage, but simply because they have descended from ancestral systems evolved for other purposes. Just as the panda's thumb is not a true digit, but a modified sesamoid bone pressed into service for gripping bamboo, some properties of our capacity for language may be better understood not as optimal



solutions to a system for communication, but as cobbled-together remnants of ancestral cognitive systems.

In language, one good candidate comes from the study of memory. According to an optimal design, if the capacity for understanding language were evolved from scratch, it would be possible to reliably retrieve individual bits of syntactic structure on the basis of their location in a hierarchical structure, independently of their content — as in most digital computers.

Instead, human language systems seem to rely on 'content-addressable' memory, a form of memory — widespread in the vertebrate world and with an apparently ancient evolutionary source — that retrieves information directly on the basis of its content, rather than through location. Unlike a computer's binary-tree structure, content-dependent memory in mammalian brains is subject to degradation over time and to interference between similar or intervening items. Human speakers are thus less likely to resolve the relation between 'admired' and 'the newspaper' in a sentence such as: "It was the newspaper that was published by the undergraduates that the editor admired," than in the briefer sentence "It was the newspaper that the editor admired." In languages such as English that lack rich case-marking, in most cases listeners can correctly interpret only two levels of embedding, not because of a strict limit on the size of representable binary trees, but because similar items become confused in memory. Although content-dependence may have its advantages, with respect to language, it clearly has its costs. A memory substrate that is scrambled by similarity and devastated by distance thus suggests that some aspects of language have

descended with modification from off-the-shelf components.

Irregular verbs (for example, go—went as opposed to walk—walked) might reflect another memory-related vestige. Although these would seem unnecessary in an ideal system, they might serve as precompiled entities to speed up sentence processing. But even then, one might expect each one to be treated as an independent entity in a table. Instead, most irregular verbs come in clusters that follow similar patterns (sing—sang, drink—drank, stink—stank, and so on). These clusters may derive from the possibility of confusion between similar items that pervades human associative memory, rather than any feature of optimal communicative design. Likewise, spoonerisms (such as referring to a loving shepherd as a shoving leopard) and other speech errors might derive from inherited limitations in human memory.

If irregular verbs, speech errors or infelicities in our abilities to parse sentences follow from ancient, inherited memory substrates, it is worth investigating whether other language properties might also follow from the properties of ancestral cognitive systems. To the extent that the neural or genetic substrates of language and cognition overlap, language should be understood not just as an adaptation selected for effective communication, but also as a darwinian descendant with modification from pre-existing cognitive systems. Studying how linguistic systems may have descended with modification from cognitive precursors could in turn elucidate the oft-noted (but never satisfactorily explained) co-morbidity between language disorders and other cognitive impairments, in terms of overlap in genetic and neural machinery. At the same time, by highlighting how new mechanisms can be built on top of old, we may be able to make better sense of the mystery of how, within a relatively short period of time, with just a relatively small amount of genetic change, humans evolved the amazing gift of speech. ■

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Neuron protection agency

Harry T. Orr

The results of an innovative way of tracing the life and death of neurons in culture favour one side of a debate about the protein accumulations associated with certain disorders of the nervous system.

On page 805 of this issue, Arrasate and colleagues¹ show that clumps of mutant protein, seen in certain neurons and characteristic of Huntington's disease, reduce the chance that the neurons will die — at least in culture. So, why is this worth noting? The answers lie in the ingenious manner in which the investigators made their observations, and because they address a point of fervent debate among those studying many human neurodegenerative diseases, including Huntington's disease.

Neuropathologists have long known that some disorders are characterized by an abnormal accumulation of macromolecules inside cells. These 'inclusion bodies' are found, for example, in the brains of patients suffering from Alzheimer's disease, prion diseases, amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), Parkinson's disease, and a group of nine so-called polyglutamine diseases, of which Huntington's disease is the most widely known. The common factor in polyglutamine diseases is a genetic mutation that produces abnormal repeats of the amino-acid glutamine in the encoded protein, with more repeats being more pathogenic.

With the advent of studies by molecular geneticists and cell biologists, it became clear that, in the inherited forms of each of these diseases, inclusion bodies contain the protein encoded by the gene containing the disease-causing mutation. These observations reignited long-standing questions of

whether and how the inclusions contribute directly to the disease process.

The debate has had especial intensity where polyglutamine diseases, and Huntington's disease in particular, are concerned^{2,3}. For the polyglutamine diseases, inclusion bodies containing mutant protein sprang to public attention in a series of papers^{2–5} that appeared in 1997. In the case of Huntington's disease, the critical protein is called huntingtin (htt), and inclusions containing mutant htt are found at the major site of neurodegeneration, the medium spiny neurons in a brain region called the striatum.

Depending on the system and the manipulations performed, over the years the experimental evidence has been interpreted as showing that the inclusion bodies cause disease, protect against disease, or are simply incidental⁶. The largest contingents are those who believe that inclusions are the major pathogenic species, owing to their ability to absorb other critical cellular proteins, and those who feel inclusions are protective, in that they sequester mutant protein out of harm's way. Resolution of this issue is necessary for many reasons, not least because many current studies aim to interfere with inclusion-body formation as a potential treatment for Huntington's disease and the other neurodegenerative disorders characterized by protein aggregates⁷.

Arrasate and colleagues¹ have devised an elegant real-time technique for assessing the factors that affect the risk of neuron death in culture. They used a common model of

Huntington's disease in which striatal neurons are transiently transfected with a pathogenic fragment of mutant htt (htt-exon1). They developed an automated microscopic system to simultaneously follow inclusion-body formation, the presence of diffuse htt and cell survival in the same neuron over a period of days. To visualize deposition of htt in transfected cells, they employed a construct of htt-exon1 fused with green fluorescent protein. As expected, inclusion bodies formed (Fig. 1), in the nucleus and in the cytoplasm, and neurons died.

To begin with, Arrasate *et al.* performed a mathematical analysis of the risk of death — a sort of actuarial assessment in tissue culture. From this analysis, they reached two conclusions. First, the risk of dying was low in neurons that had been transfected with a control (non-pathogenic) form of htt-exon1 and high in cells expressing htt-exon1 with an expanded polyglutamine tract; furthermore, the risk increased with increasing size of the mutant polyglutamine stretch. Second, they found that the risk of death in cells with mutant htt-exon1 was linear over time; that is, risk seemed to be largely time-independent.

So, what variables affected the risk of cell death? Not unexpectedly, inclusion bodies were one such variable. Importantly, the basic features of inclusion-body formation — size and growth with time — in this model system accurately replicated the picture seen in Huntington's disease. By following the same neuron over time, Arrasate *et al.* found that cells that failed to form inclusion bodies

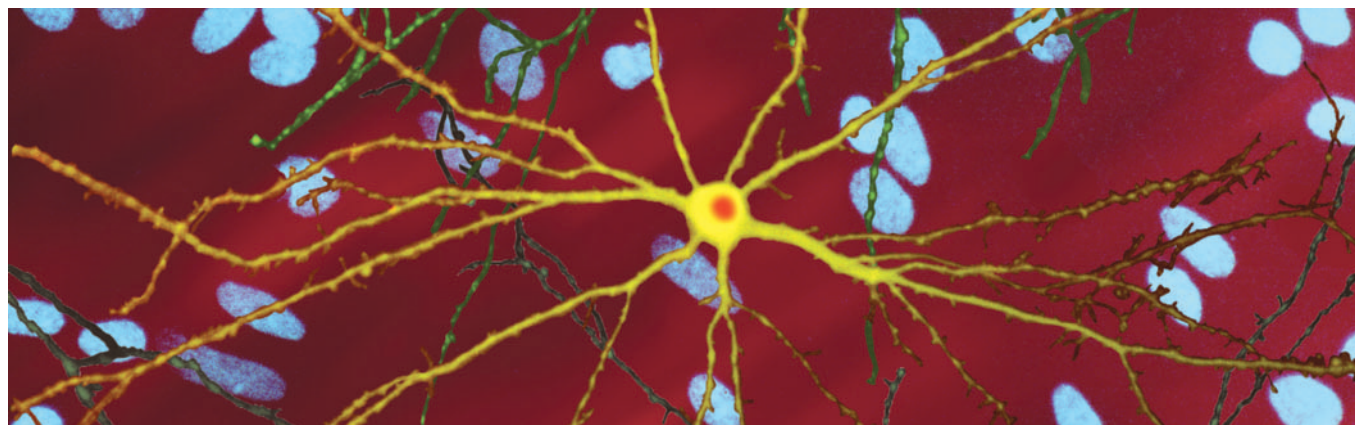


Figure 1 Picture of health? This image, produced by Arrasate *et al.*¹, shows a striatal neuron (yellow) that has been transfected with the disease-associated version of huntingtin, the protein that causes Huntington's disease. The orange–red structure is an inclusion body.

Arrasate *et al.* show that transfected neurons that form such bodies live longer than those that do not. Nuclei of untransfected neurons (blue) are seen in the background, along with projections from other neurons (green).

had an increased risk of death, indicating that the inclusion body is not required for polyglutamine-induced neuronal death. Remarkably, when neurons expressing equal levels of mutant htt-exon1 were followed individually, those neurons in which inclusion bodies formed had a significantly reduced risk of dying compared with neurons in which mutant htt-exon1 remained diffuse. Moreover, inclusion-body formation reduced the amount of diffuse mutant htt elsewhere in the neuron.

So inclusion-body formation actually prolonged survival and protected neurons, seemingly by reducing the amount of a toxic, diffusely distributed form of mutant htt. But although the results provide compelling evidence that readily visible ($1\ \mu\text{m}^2$), mutant polyglutamine inclusion bodies are protective, and not pathogenic, they do not rule out the possibility that the major toxic species are the early precursors to inclusion bodies —

typically referred to as microaggregates.

The long-term strength of this study¹ lies in the approach itself: the ability to find out whether a cellular feature of a disease is pathogenic, beneficial or merely incidental will help greatly in understanding disease mechanisms. It will also be interesting to see whether the results end the debate on the pathogenic role of inclusion bodies in polyglutamine diseases. If they don't, one wonders what would.

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Nuclear physics

Neutron halo slips

David Hinde and Mahananda Dasgupta

In neutron-rich nuclei, weakly bound neutrons form a halo surrounding a compact core. Unexpectedly, it seems that this halo does not improve the chances of the nucleus fusing with another nucleus.

Accelerator facilities that can supply beams of highly unstable, radioactive isotopes are making it possible to study nuclear reactions that occur naturally only in violent cosmic events such as supernovae, but which determine many of the elemental and isotopic abundances found on Earth. Some of the most neutron-rich of these nuclei have a diffuse neutron cloud that extends to large distances beyond the compact nuclear core. Based on the lessons learned from the fusion of stable nuclei, this halo might be expected to enhance, many times over, the probability of nuclear fusion at low energies. But, on page 823 of this issue, Raabe and colleagues¹ describe an ingenious measurement that shows no such enhancement, indicating that the behaviour of the neutron halo is more unusual than expected.

Atomic nuclei, which are made up of protons and neutrons, exist because of the attractive nuclear force between their constituents. This force depends on many variables, but in particular favours equal numbers of protons and neutrons. Thus, for example, the most common isotopes of the elements helium and nitrogen, having two and seven protons respectively, are ^4He and ^{14}N . If more neutrons were added to a nucleus, either one by one, or in pairs, the nucleus would become less strongly bound. Eventually, at the point where the energy of the new nucleus is greater than that of its

'ingredients', the nucleus would become unbound. The transition from bound to unbound neutron-rich nuclei occurs at the so-called neutron drip-line, which marks the boundary of the existence of nuclei (Fig. 1).

For the examples of helium and nitrogen, the drip-line isotopes are ^8He and ^{23}N . These and other neutron-rich nuclei are not found on Earth because they undergo β -decay, in which a neutron is transformed into a proton, improving the balance between neutrons and protons.

Nuclei close to the neutron drip-line can have one or two extremely weakly bound neutrons. The energy required to separate the two weakly bound neutrons from ^6He is only 0.973 MeV (megaelectronvolts), compared with typically 8 MeV to remove a single neutron from a stable nucleus. The low binding energy of these neutrons results in quantum-mechanical tunnelling to large distances, allowing their wavefunctions (or probability distributions) to extend well beyond the tightly bound core, forming a diffuse neutron cloud or halo (Fig. 1).

The effect that this halo might have on nuclear fusion has been the subject of some controversy. Because the nuclear force has a short range, the attractive force between two nuclei is closely related to the degree of overlap of their matter distributions. The potential barrier (fusion barrier), which must be overcome in fusion, occurs at the radial separation where this force balances the repulsive Coulomb force between the positively charged protons in the two colliding nuclei. According to this picture, the neutron halo might be expected to contribute a longer-range attractive force, which would reduce the energy of the fusion barrier. Furthermore, in reactions between stable nuclei, the likelihood of fusion occurring is known to be enhanced at energies well below the fusion barrier if the transfer of one or more

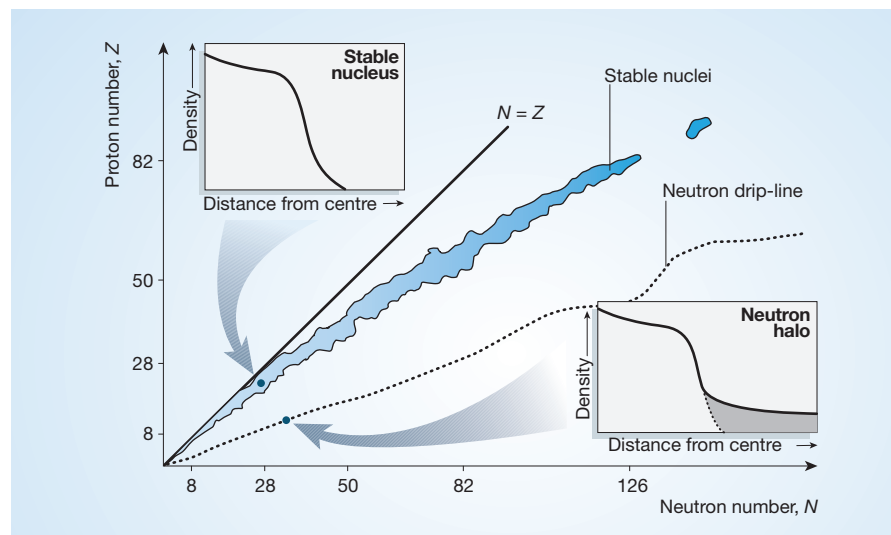


Figure 1 Nuclear stability and neutron halos. In stable nuclei, the number of neutrons tends to exceed the number of protons. Nuclei with too many neutrons, however, are unstable; beyond the 'neutron drip-line', nuclei become unbound. Isotopes close to this line may have one or two neutrons that are weakly bound to the core nucleus. Through quantum-mechanical tunnelling, and because their binding energy is low, these neutrons form a nuclear halo: the neutron density extends to greater distances (inset, right) than is the case in a well-bound, stable nucleus (inset, left). According to Raabe et al.¹, the existence of this neutron halo does not make it any more likely that the nucleus will undergo fusion.

Optical fibres

A light fabric

It might seem an unlikely addition to the world of fashion design, but the creators of this new fabric (right) claim that it is wearable. Mehmet Bayindir and colleagues have melted together strands of optical, metallic and insulating materials to form fibres that are then woven into what they call a "spectrometric fabric" (*Nature* **431**, 826–829; 2004).

Each fibre has separate channels for transmitting light and electrical currents, and these affect each other in subtle ways to create an intricate web of optical and electrical patterns.

The fabric would make curious clothing indeed, but it serves to demonstrate the level of sophistication reached in the design of optical fibres. We are all familiar with their use in high-speed telecommunication networks, but the past decade has seen the development of a new range of optical fibres with periodic structures along their length that are on the scale of the wavelength of the light transmitted. This microstructure dramatically affects the way the light is guided through the fibres. By choosing the right



pattern, fibres can be made to transmit a certain range of wavelengths, or optical pulses with specific shapes. Such tailored properties could be used, for example, in beam delivery for medical or industrial laser applications, or even in the all-optical processing of data.

Bayindir *et al.* go a step further and consider what fibre-based applications could benefit from both

electronic and optical functionality.

The main challenge is to find the right combination of metallic, insulating and semiconductor materials that can be drawn together into metres of fibres with continuous structures along their entire lengths. Using a low-melting-point material such as tin for the metal parts is crucial, the authors report.

They have come up with an

application that makes the most of all the favourable features of these new fibres: a two-dimensional photodetector based on an interwoven grid. The fibres generate electrical currents when they sense light anywhere along their length, and the grid geometry provides an effective means of determining the exact location of an illuminating spot.

Liesbeth Venema

neutrons increases the total binding energy of the system². This is always the case in reactions between a halo nucleus and a stable nucleus. In contrast to these fusion-enhancing mechanisms, it has been demonstrated that even stable nuclei, if weakly bound, can break up before reaching the fusion-barrier radius, resulting in a suppression of complete fusion if one or more of the fragments escape the target nucleus³. So, is the probability of fusion in reactions involving halo nuclei enhanced or suppressed compared with reactions involving non-halo isotopes?

In an earlier experiment that compared the reactions of ^4He and ^6He nuclei when fired at a uranium (^{238}U) target, much-increased product yields — attributed to fusion — were found for ^6He , at energies below the fusion barrier⁴. But were these large yields really due to fusion, or to other processes? Fusion with a target nucleus of ^{238}U results in fission, which is used as a robust signal of the preceding fusion. However, fission can also result from the transfer of neutrons from ^6He to ^{238}U . Raabe and colleagues¹ have disentangled these two processes, by looking both for fission fragments and for the ^4He core that remains

following neutron transfer. Fission events that are detected in coincidence with ^4He nuclei are attributed to transfer reactions; those with no coincidence are attributed to complete fusion. Raabe and colleagues' data provide a clear demonstration that the large fission yields below the fusion barrier do not result from fusion with ^6He , but from neutron transfer. This conclusion is in line with other measurements^{5,6} that have identified large probabilities for neutron transfer in the reactions of ^6He and ^8He with lighter target nuclei — copper, zinc and osmium.

Quantum-mechanical calculations by Nakatsukasa *et al.*⁷ offer some insight into what is happening to the halo during the collision. They have modelled the behaviour of the halo neutron of the beryllium isotope ^{11}Be in collisions with another heavy nucleus, the lead isotope ^{208}Pb . They find that the deceleration of the ^{10}Be core in the Coulomb field of the ^{208}Pb nucleus is sufficient for there to be a substantial probability that the halo neutron will be torn from the ^{11}Be nucleus. If the nuclear attraction between the halo neutron and the ^{208}Pb nucleus is also taken into account, that would further enhance the probability of transfer. In the reaction studied

by Raabe *et al.*¹, the energy gained through the transfer of the two halo neutrons to the ^{238}U target nucleus is much larger still, so transfer should be even more favourable. The weak coupling of the halo neutrons to the ^4He core, together with the wide extent of the halo, leads to a picture in which the halo neutrons can be grabbed from the ^6He nucleus by the ^{238}U nucleus when the two are still some distance from each other. Thus at smaller separations, close to the fusion-barrier radius, there is little chance of this transfer assisting the fusion process.

A strange new picture is emerging of the interaction of the neutron halo. We speculate that a largely sequential process occurs. The halo extends to such a large radius, and its coupling to the target nucleus is so strong, that the nuclei finally coming into contact at the fusion-barrier radius are most likely to be the core of the projectile nucleus and a target nucleus that has already 'eaten and digested' the halo neutrons. Thus there is little or no fusion of the halo nucleus itself but — and this is novel — we have fusion of the projectile core with a more neutron-rich, possibly unstable, excited target nucleus.

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Circadian rhythms

Sunrise and sunset in fly brains

William J. Schwartz

Fruitflies can time their morning and evening activities to the day–night cycle. The basic circadian oscillatory mechanism is intracellular, but networks of cells, now being identified, are what make a working clock.

Animals have an internal timekeeping mechanism that precisely regulates 24-hour (circadian) rhythms of body function and behaviour, and synchronizes them to the day–night cycle. A constellation of ‘clock’ genes lies at the core of this timepiece, and these genes interact in complex intracellular feedback loops to produce oscillations in their own expression¹. But how are such molecular cycles translated into the adaptable temporal programmes that are characteristic of whole organisms? In fruitflies (*Drosophila melanogaster*), for example, how does a daily intracellular molecular oscillation drive a rhythm of rest and activity that is overtly bimodal, with pronounced bouts of activity around morning and evening that can anticipate the times of lights-on and lights-off? On pages 862 and 869 of this issue, Stoleru *et al.*² and Grima *et al.*³ show that such behaviour arises at an intercellular (tissue) level of organization, with discrete sets of clock-gene-expressing brain cells being differentially involved in the response to dawn and dusk.

One well-known clock gene in fruitflies is *period* (*per*), which, in the brains of adult flies, is expressed in photoreceptor cells, glial (non-neuronal) cells and in a few clusters of neurons⁴. These neuron clusters lie in specific areas of the brain: there are three groups of dorsal neurons (DN₁, DN₂ and DN₃), and two groups of lateral neurons, on each side of the brain (Fig. 1). One group of five to eight lateral neurons lies towards the top of the brain (that is, dorsolaterally; these are called LN_d neurons). The other group, the LN_v neurons, lies towards the bottom of the brain (ventrolaterally); it includes four to six large cells and five small cells. The LN_v neurons (except for one of the five small cells) express the neurotransmitter molecule known as pigment-dispersing factor (PDF), whereas none of the LN_d or dorsal neurons does.

Attention has focused on the LN_v neurons as the essential circadian ‘pacemaker’ cells that set fly activity rhythms, especially so because removing them leads to defective

behavioural rhythmicity in constant darkness^{5,6}. But data from work on flies lacking PDF and on other mutants, as well as studies in which flies were engineered to express neuronal genes that block electrical activity or synaptic transmission, suggest that a multi-neuronal network is also somehow involved^{7–11}.

Stoleru *et al.*² and Grima *et al.*³ sought to dissect this network, using mutant flies and clever genetic crosses to target specific genes to specific cells. Thus, Stoleru *et al.* succeeded in delivering a cell-death gene to the LN_v or LN_d neurons in flies, killing these cells. Tests of the flies’ cycles of rest and activity showed that the insects’ behaviour was still rhythmic in a light–dark cycle — but the rhythms were different. The LN_v-lacking flies anticipated only lights-off in the evening, not lights-on in the morning. Meanwhile, the flies nominally lacking LN_d anticipated lights-on but

not lights-off. In constant darkness, the strains showed unimodal evening- or morning-phased rhythms, respectively (although rhythmicity could not be sustained in the LN_v-less flies).

Grima *et al.* used a different, non-ablative strategy. They started with flies that were deficient in *per* and therefore arrhythmic, and then forced the re-expression of *per* only in the LN_v neurons, or in both the LN_v and LN_d neurons. LN_v-restricted *per* expression rescued behavioural rhythmicity, but only lights-on was anticipated (actually, expression in the small LN_v cells seemed to be sufficient for this, even in constant darkness). Lights-off was also anticipated when *per* was also expressed in about half of the LN_d cells. Thus, two independent strategies have led to the same conclusion: morning and evening bouts of activity are differentially controlled by the LN_v and LN_d cells, respectively.

There was in fact already evidence that morning and evening bouts of locomotor activity in flies are each governed by their own circadian oscillators¹², but the neural mechanisms involved were unknown. In 1976 it was proposed¹³ that circadian rhythms in rodents are generated by a complex pacemaker consisting of two mutually coupled circadian oscillators. These consist of a morning oscillator (*M*) accelerated by light and synchronized to dawn, and an evening oscillator (*E*) decelerated by light and synchronized to dusk. The circadian pacemaker that regulates rodent locomotor rhythmicity — the suprachiasmatic nucleus in the hypothalamus of the brain — contains a mixed neuronal population¹⁴, and there is some evidence that it is composed of two oscillating *M* and *E* components¹⁵. The neural elements

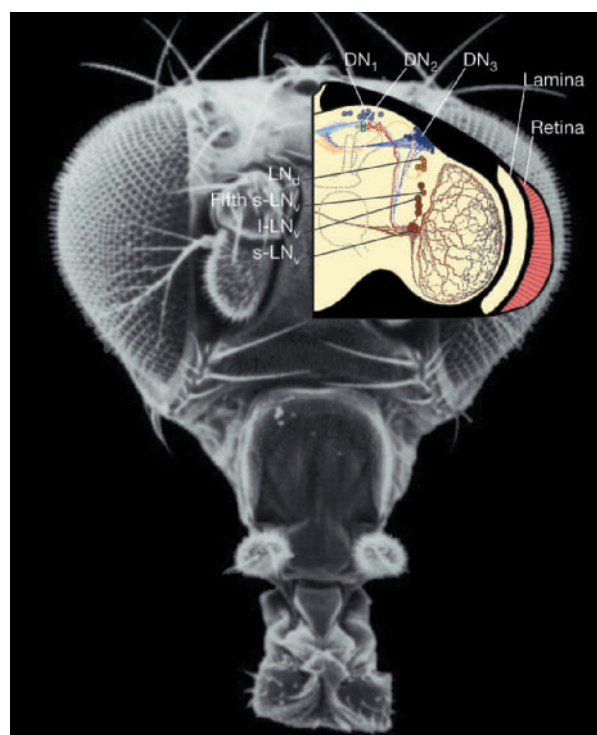


Figure 1 Clock-gene-expressing neurons and their outputs in the fruitfly brain. These nerve cells include, on each side of the brain, three groups of dorsal neurons (DN₁, DN₂ and DN₃) and two groups of lateral neurons, one dorsolateral (LN_d) and the other ventrolateral (LN_v; either large, l, or small, s). The fifth small LN_v cell is different from the other LN_v neurons in that it does not express the neurotransmitter molecule pigment-dispersing factor. The new papers^{2,3} show that the LN_v and LN_d cells differentially control morning and evening bouts of locomotor activity, respectively. (Diagram courtesy of C. Helfrich-Förster, Univ. Regensburg, Germany.)

involved are unknown, but the LN_v and LN_d neurons are now plausible candidates for two analogous oscillators that control the morning and evening bouts of activity in flies.

With the flies engineered by Stoleru, Grima and their colleagues, clock researchers are in a position to rigorously test dual-oscillator hypotheses. For example, the phase shifts shown by the insects in response to differing light pulses can be analysed, as can their period changes in constant light of varying intensity, and their response to altered day length (photoperiod). There should be much to learn about the network interactions of clock genes, cells and outputs. For instance, the molecular oscillations within LN_v and LN_d cells seem to exhibit a similar phase despite the cells' differing roles, and it seems likely that additional neuronal groups, such as the DN cells, are also involved¹⁶. Furthermore, a network of interconnected neurons could generate an oscillatory output without requiring every neuron to be independently rhythmic. Indeed, Stoleru and colleagues² found that driving *per* re-expression in *per*-deficient flies in all putative clock neurons except the PDF-expressing LN_v cells (that is, in DN, LN_d and the fifth small LN_v cells) could still restore the anticipation of both lights-on and lights-off.

Of course, flies can do much more than merely walk about in a tube, and activities such as olfaction and reproduction (in individuals), as well as hatching (in populations), also show circadian rhythmicity¹. Other studies have found that peripheral tissues that display molecular oscillations are part of the underlying circadian circuitry, and that their organization is complex, with some tissues (such as the prothoracic gland in hatching¹⁷) depending on LN_v input, and others (such as the antennae in olfaction¹⁸) being apparently autonomous. A truly integrative circadian biology is close at hand, as researchers learn about an adaptable, layered system that has emergent properties at many levels of organization. *Drosophila* workers, who have been so effective at taking the clock apart, are now succeeding in putting it back together. ■

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Neurobiology

Accessing a transporter structure

Michael P. Kavanaugh

Information processing in the brain requires the neurotransmitter glutamate. Hence the importance of today's publication of the structure of an archaeal relative of the transporter controlling glutamate's levels.

In the brain, neurons communicate with each other primarily through the use of neurotransmitters. These chemical signals are released by presynaptic neurons in response to electrical impulses, then detected and converted back into electrical signals by receiving (postsynaptic) neurons. For this process to allow recurrent and selective signalling, the neurotransmitter must be efficiently removed soon after release¹. On page 811 of this issue, Yernool and colleagues² describe the structure of an ancestral counterpart of the membrane transporter that is responsible for clearing glutamate — the predominant excitatory neurotransmitter in the mammalian central nervous system. This structure is the first to be obtained for a neurotransmitter-transporter-related protein. It is also the first amino-acid-transporter structure to be solved.

In a sense, this work marks the end of a chapter that began decades ago, with the discovery of a transport system that uses the gradients of ions across the plasma membranes of brain cells to concentrate glutamate within them^{3–5}. The molecular species responsible for this activity were subsequently identified as members of a mammalian family of five genes that encode glutamate transporters⁶. Now, with the structure and model presented by Yernool *et al.*², we receive some tantalizing new clues to how these proteins work — and so this publication

also marks the beginning of a new chapter in the study of their structure and mechanism.

The glutamate-transporter family had previously been recognized as being distinct from the larger family of transporters for other neurotransmitters, such as dopamine, noradrenaline, serotonin and γ -aminobutyric acid. Proteins in the latter family, and in the very large 'major facilitator' superfamily of transporters (MFS)⁷, each have a topology that consists of 12 membrane-spanning α -helical domains (transmembrane domains, TMDs), connected by intracellular and extracellular loops. But the topology of glutamate transporters has been more controversial, complex, and difficult to elucidate. Although they seemed to have eight TMDs that behave as membrane-spanning α -helices, biochemical analyses⁸ suggested that the carboxy-terminal region also contains two hairpin loops — located between TMDs 6 and 7 and between TMDs 7 and 8 — that partly re-enter the membrane from opposite sides (Fig. 1a).

This carboxy-terminal region — including the 're-entrant' loops and TMDs 7 and 8 — has been a focus of study because it contains interesting functional determinants, including sites that interact with glutamate and competitive analogues, and with key ions such as sodium and potassium (reviewed in ref. 6). A stoichiometric coupling mechanism, in which three Na^+ ions

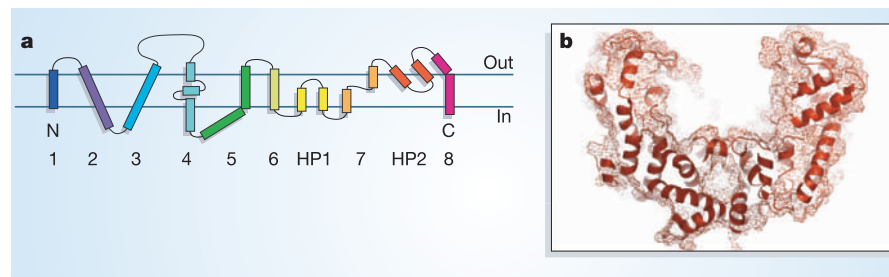


Figure 1 Glutamate transporters in two and three dimensions. a, Schematic topology of the archaeal glutamate-transporter-related protein Glt_{ph} (ref. 2) — showing the transmembrane domains (1–8) and the two 're-entrant' hairpin loops (HP1 and HP2). N, amino terminus; C, carboxy terminus. b, Side view of a Glt_{ph} trimer in the membrane plane. One of the three Glt_{ph} subunits is removed in this view to reveal a bowl-like structure.

D. YERNOOL ET AL.

and one proton are transported across the membrane with glutamate and one K^+ ion is transported in the opposite direction, allows a gradient of glutamate to be maintained such that the extracellular concentration is up to a million times lower than the intracellular concentration. This is necessary because prolonged activation of glutamate receptors on the extracellular surface of nerve cells is toxic — and high concentrations of glutamate are already present within the cells⁶.

A common theory invoked to explain flux coupling in glutamate and other transporters proposes a conformational transition that allows alternating access to the transporter by intracellular and extracellular substrates⁹. But how do transporters accomplish this in a manner that prevents substrates from simply diffusing through the transporter down their electrochemical gradients? To understand this, we need to elucidate the nature of the 'gating' machinery involved. The structure and molecular model discussed by Yernool *et al.*² go some way towards providing both explanations and testable predictions.

The authors solved the structure of a transporter, Glt_{ph}, from the thermophilic archaeal species *Pyrococcus horikoshii*, and refined it to a resolution of 3.5 Å to reveal a wealth of interesting features. This protein shares more than 35% amino-acid sequence identity with human glutamate transporters, but an important caveat is that glutamate-transport activity has not yet been demonstrated for this archaeal transporter. This may relate to special temperature requirements, or to modulation by specific lipids as exhibited by some bacterial (and mammalian) glutamate transporters⁶. Nevertheless, signature sequences and elements of the three-dimensional structure of Glt_{ph} are consistent with a large body of biochemical and biophysical data for mammalian glutamate transporters. Furthermore, an electron density consistent with a bound molecule of glutamate, which was present during crystallization, is found nestled between the re-entrant hairpin loops, where it is poised to interact with key amino acids in TMDs 7 and 8 (ref. 2).

The folding pattern seen in the structure is distinct from that seen for the other transporters, pumps and channels that have been crystallized so far. The existence of the re-entrant loops is now confirmed. A provocative and attractive model put forward by Yernool *et al.* is that these hairpin loops, HP1 and HP2, serve as the gates controlling intracellular and extracellular access, respectively. This mechanism is quite distinct from the 'rocker-switch' type mechanism suggested by the MFS structures⁷. Coordinated movement of the loops could allow alternating access without an 'open channel' state. The state crystallized in the presence of glutamate

is consistent with its being occluded, with the loops closed upon the substrate. Movements of these gates would be consistent with biochemical and fluorescence data that suggest that the protein's conformation changes in a state-dependent fashion when glutamate and ions bind^{6,8,10}.

Curiously, a chloride-channel activity is also observed in glutamate transporters, although chloride flux is not stoichiometrically coupled to that of glutamate⁶. Amino acids in the surrounding amino-terminal helices, especially helix 2, have been implicated in the function of this channel¹¹, but details of its location and mechanism of gating await further data.

Another interesting feature of the transporter is its trimeric structure², confirming previous experimental suggestions⁶ of a multimeric nature for glutamate transporters. Yernool *et al.* have tentatively identified three binding sites for glutamate, one in each subunit, hinting that each subunit might function independently. So why is the transporter multimeric? That remains to be seen. The structure shows that three wedge-shaped subunits assemble to form a bowl-like structure, in which the basin faces the extracellular solution and the smaller base faces the cytoplasm (Fig. 1b). The deep, hydrophilic surface of the basin interior dips far into the plane of the membrane and may provide an aqueous 'waiting area' for transported solutes. But the role of this feature awaits more information, including the precise entry path for glutamate and ions into each subunit.

Even more crucial is the need for information about the conformational transitions that occur before and after ion and glutamate binding — transitions that are central to the alternating-access hypothesis. The new structure and model² are not the final word, but they suggest some promising experimental paths towards a satisfying explanation of how transporters work at the submolecular level. ■

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100 YEARS AGO

As a constant reader of *NATURE* and of papers read before scientific societies, I have been struck by what seems to me an inaccurate use of language by English men of science which is rarely chargeable upon Americans — which is, at any rate, at variance with American usage. I will illustrate with the following examples:—One star is five light-years distant; another is twenty-five light-years distant. The English astronomer will say that the second is *five times farther away* than the first. A mass of aluminium weighs one pound; a mass of lead of equal size weighs something more than four pounds. The English physicist will say that the aluminium is *more than four times lighter* than the lead. Both expressions seem to me incorrect and unworthy of a man of science who endeavours to express himself accurately. In the one case he should say that one star is five times *as far away* as the other. In the other case the whole expression is vicious. Weight, heaviness, is an attribute of matter; lightness is absence, or deficiency, of weight. To say that one article is a certain number of times *lighter than* another is like saying of two vessels unequally exhausted of air that one is four times emptier than another. It is good English — is it not? — to say that one article is twice as heavy as another. If it is twice heavier, it is three times as heavy. I submit this criticism of an Anglicism as an offset to some one of many criticisms of Americanisms.
E. S., Boston, U.S.A.
From *Nature* 13 October 1904.

50 YEARS AGO

On September 6, President Eisenhower announced that agreement had been reached between the United States and six other nations to establish an international agency which would foster the growth and spread of the new atomic technology for peaceful purposes. Atomic materials would be set aside for projects sponsored by the agency, and when arrangements were complete the United States would establish a reactor school to train representatives of friendly nations in the skills needed for their own atomic purposes... Mr Dulles, the American Secretary of State,... indicated that it was now proposed to create an international agency the initial membership of which included nations from all regions of the world... No nation would be excluded from participation in this venture.
From *Nature* 16 October 1954.

Obituary

Jacques H. van Boom (1937–2004)

Jacques van Boom, who died on 31 July, is best known for his seminal work in synthesizing strings of nucleic acids of defined sequence. Such oligonucleotide sequences are now made and delivered with a 24-hour turnaround time, but it wasn't always so. In the late 1970s, workable methods for producing them were few and far between, especially for the large quantities required for structural studies. Much of the revolution in biology that stems from techniques such as the polymerase chain reaction, or site-directed mutagenesis, would have been impossible without easy access to oligonucleotides of defined sequence.

More generally, much of the chemistry that has found its way into applications now considered routine in the life sciences has its roots in the pioneering work of chemists such as van Boom — the synthesis not only of nucleic acids, the building-blocks of DNA, but of chains of peptides and sugars (oligo- and polysaccharides) as well.

Van Boom was born in 1937 in Simpelveld, in the Netherlands. He studied chemistry at Utrecht University, obtaining his PhD there in 1968. Following postdoctoral training in the laboratories of Lord Todd and C. B. Reese at the University of Cambridge, he turned his attention to the development of ways of synthesizing nucleic acids. Following a move to Leiden University, where he became a professor in 1975, he developed a new series of phosphorylation techniques as essential intermediates for the synthesis of oligonucleotides. The most prominent of these approaches was his modification of the so-called phosphotriester method; although van Boom's methods have been superseded by phosphoramidite chemistry, they did allow the synthesis of pure oligonucleotides on solid supports, and in automated fashion in amounts that could be studied structurally.

One such oligonucleotide, CGCGCG, a self-complementary hexamer, became the subject of a highly successful collaboration, begun in the late 1970s, between van Boom and Alex Rich, at the Massachusetts Institute of Technology. With milligram quantities of the pure oligonucleotide to hand, Rich and his colleagues succeeded in obtaining crystals that diffracted to 0.9 Å, yielding a structure of the oligonucleotides at atomic resolution.

Here it needs to be remembered that although the famous Watson–Crick model



Pioneering studies in bio-organic chemistry

of DNA, based on fibre diffraction patterns obtained by Rosalind Franklin and Maurice Wilkins, was consistent with the data then available, it lacked the atomic resolution of the single crystal analysed by Rich and van Boom. As Rich tells the story, even though every atom in the six-base-pair structure was visible, the structure itself could not be modelled as the typical B (right-handed helix) form of DNA, but rather surprisingly revealed itself as a left-handed helix. Francis Crick, apprised of this fact by Rich ("Francis, we have the structure, and it's a left-handed helix!"), was nonplussed — until Rich pointed out that van Boom's oligonucleotide assumed a conformation previously inferred from circular-dichroism data obtained for DNA in high salt concentrations, as an alternative ('Z' form) to the more common B form of DNA seen with low salt.

Refining his synthetic methods to include modified nucleotides, van Boom, with Sidney Hecht, went on to work out the mode of action of the DNA-targeting antibiotic bleomycin.

Van Boom's more recent work extended to the synthesis of peptide–nucleotide molecules, which (with Eckard Wimmer) in 1998 were used successfully to unravel the replication mechanism of poliovirus. This required the synthesis of homogeneous peptides with uridyl groups attached, and necessitated a successful joining-at-the-hip of peptide and nucleic-acid chemistry.

The automated, solid-phase synthesis of oligosaccharides of predetermined length

and structure has, however, been a much tougher nut to crack. Nonetheless, an early highlight in the area of glycobiology was the development of a synthetic vaccine against *Haemophilus influenzae* type b, which causes pneumonia and meningitis. A synthetic conjugate vaccine against this bacterium, produced essentially by the route reported by van Boom, is effective and in use.

In the early 1990s, van Boom worked out a set of activator systems that turn sugars containing sulphur (thioglycosides) into effective donors for oligosaccharide assembly because of their intrinsic stability under many synthetic conditions. With the development of suitable promoter systems, thioglycosides are now used as donor or acceptor in oligosaccharide synthesis and have facilitated the synthesis of many oligosaccharides and conjugates between sugars and other molecules, both in solution and on solid supports. Van Boom's description of the thioglycoside method, published in *Tetrahedron Letters* in 1990, is one of the most cited papers in modern carbohydrate chemistry. Applications include the industrial-scale fabrication of heparin analogues to control blood clotting.

The Netherlands is a small country with a small scientific community, and van Boom's passion for science was at times mistaken for brusqueness and an unwillingness to get involved. Quite simply, however, he had an aversion to any activity that did not involve science itself. He distilled most of the commonly used solvents himself, an activity that not only saved his students' time and the lab money, but also provided him with a ready excuse to leave meetings he considered useless. His preferred mode of transport, a moped, sometimes used even in the lab corridors, similarly allowed him to claim mechanical breakdown as a means of avoiding unproductive meetings. His brutal honesty in scientific matters was paired with a *joie de vivre* more typical of the Burgundy-oriented southern Netherlands than the damp and puritanic north.

A heavy smoker, he died of lung cancer. He leaves behind his wife Liesbeth, a daughter and two grandchildren.

Gijs van der Marel and Hidde Ploegh

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Quantum optics

Setting our watches by entanglement

Appl. Phys. Lett. **85**, 2655–2657 (2004)

Synchronizing distant clocks is a crucial process for telecommunications and satellite positioning systems. It is also important for high-accuracy experiments in fundamental physics, such as tests of the theory of relativity. Achieving synchronization can sometimes be the limiting factor in the accuracy of such measurements.

Alejandra Valencia *et al.* have conducted a proof-of-principle experiment showing that quantum entanglement of photon pairs can provide a synchronization method that avoids the shortcomings of existing techniques. By sending two entangled photons (produced by shining laser light into a nonlinear optical material) down optical fibres 1.5 km long, they have been able to detect synchronized events with picosecond (10^{-12} s) accuracy over a distance of 3 km.

For an entangled pair of particles, a measurement on one of them instantaneously determines the quantum state of the other. To use this for synchronization, the two photons are dispatched to detectors at the two 'stations' where the clocks are to be synchronized — say, a satellite and an Earth-based laboratory. If the clocks used to time the detection of many successive pairs are synchronized, the event timings will show the greatest number of coincidences when the records are compared. In cases of mismatch, the clocks can be altered until the detections coincide.

Philip Ball

Developmental biology

Timing is everything

Cell **119**, 87–96 (2004)

When fruitfly larvae are starved, they temporarily stop growing — but researchers have been unsure how they also freeze the carefully timed differentiation of their tissues. Joseph M. Bateman and Helen McNeill show that the same pathway that halts growth also arrests differentiation.

Bateman and McNeill studied the developing eye, whose light-receptor cells differentiate in a carefully regulated wave that moves across the tissue. The authors found that cells lacking a working copy of the gene *tuberous sclerosis complex 1* (*tsc1*) differentiate several hours prematurely.

The protein TSC1 works in a pathway of molecules that is triggered by the insulin receptor, which signals whether sufficient nutrients are available and also controls the fly's rate of growth. The authors went on to show that other mutations that boost

signalling from the insulin receptor also accelerate differentiation. Conversely, mutations that cut insulin-receptor signalling (as nutrient deficiency would also do) delay differentiation.

The researchers propose that this pathway of molecules helps the organism to tightly coordinate its growth and differentiation with its nutritional status. And because mutations in TSC1 seem to hasten differentiation, it might also explain why human tumours that have lost a working copy of the *TSC1* gene contain highly differentiated tissues.

Helen Pearson



Biophysics

Flies get a grip

Proc. R. Soc. Lond. B doi:10.1098/rspb.2004.2850 (2004)

How does the fly that's been annoying you by crawling up your window manage to hold on? A study of fly footprints shows that the offending insect relies mainly on capillary forces generated by fluid secreted from its feet.

Mattias Langer *et al.* used atomic-force microscopy to examine the adhesiveness of tiny puddles of foot fluid left by a fly, *Calliphora vicina*, as it walked across a glass slide. As the fluid evaporates, its stickiness decreases, showing that the fluid plays an important role in generating adhesion between foot and substrate. Adhesion measured in air was much stronger than that measured in an aqueous environment, indicating that capillary forces are mainly involved in the fly's attachment mechanism.

Electron-microscopic examination of the fly's feet supports this theory. The attachment pads at the end of the insect's legs are covered with tiny bristles called setae, each ending in a spatula-shaped terminal plate. Coated with fluid secreted from the centre of the attachment pad and delivered through a channel to the terminal plate, the plate is perfectly shaped to form a contact with the substrate through this capillary bridge.

Michael Hopkin

Nanoparticles

Safer fullerenes

Nano Lett. doi:10.1021/nl0489586 (2004)

One of the main concerns about nanotechnology is the potential toxicity of nanoparticles. A study earlier this year suggested that colloidal aggregates of carbon-60 fullerene molecules (C_{60}) can cause oxidative damage to lipids in the brains of fish (E. Oberdorster *Environ. Health Perspect.* **112**, 1058; 2004) — the first real indication that fullerenes could be toxic. Such clusters of C_{60} could find their way into groundwater and streams if fullerenes were to attain some of their predicted applications in consumer products.

Christie M. Sayes *et al.* have now conducted *in vitro* studies of fullerene toxicity in human cells, and find that nC_{60} causes lipid oxidation and cell death there too, through the molecules' ability to generate oxygen free radicals.

But it's not all bad news. Chemically modified fullerenes can be far less cytotoxic: fullerenes with carboxylate or hydroxyl groups attached were much less harmful. These chemical groups make the molecules a lot more soluble in water, and the more of them there are, the less toxic is the compound: $C_{60}(OH)_{24}$ showed no cytotoxicity even for the maximum concentration that its solubility permits.

Philip Ball

Stem cells

Making a prostate

Development **131**, 4955–4964 (2004)

The prostate gland in rams grows and shrinks as concentrations of testosterone rise and fall, leading many researchers to assume that this gland (in sheep and other species) must contain stem cells that can sprout new tissue. But there has been controversy as to whether these elusive stem cells lie in the luminal epithelium, which pumps out seminal fluid, or in the basal epithelium that supports the luminal layer.

Takeshi Kurita *et al.* find that the prostate gland can regrow in the absence of the basal layer. They examined mouse embryos that had been genetically engineered to lack the *p63* gene and which had no basal cells as a consequence. When the authors grafted the budding prostate tissue from these embryos into adult mice, they found that it could produce most cell types apart from basal cells. When the mice were castrated and their testosterone levels plummeted, the prostate tissue shrivelled up, but was able to regenerate when testosterone was restored.

The results suggest that the luminal epithelium contains putative stem cells that can give rise to most cell types in the prostate, and that basal cells direct their differentiation.

Helen Pearson

Structural plasticity in the bilingual brain

Proficiency in a second language and age at acquisition affect grey-matter density.

Humans have a unique ability to learn more than one language — a skill that is thought to be mediated by functional (rather than structural) plastic changes in the brain¹. Here we show that learning a second language increases the density of grey matter in the left inferior parietal cortex and that the degree of structural reorganization in this region is modulated by the proficiency attained and the age at acquisition. This relation between grey-matter density and performance may represent a general principle of brain organization.

We used a whole-brain unbiased objective technique, known as voxel-based morphometry (VBM)^{2,3}, to investigate structural plasticity in healthy right-handed English and Italian bilinguals.

To test for differences in the density of grey and white matter between bilinguals and monolinguals, we recruited 25 monolinguals who had had little or no exposure to a second language; 25 'early' bilinguals, who had learned a second European language before the age of 5 years and who had practised it regularly since; and 33 'late' bilinguals, who had learned a second European language between the ages of 10 and 15 years and practised it regularly for at least 5 years. All volunteers for this test were native English speakers of comparable age and level of education.

Voxel-based morphometry revealed that grey-matter density in the inferior parietal cortex was greater in bilinguals than monolinguals (Fig. 1a). This effect was significant in the left hemisphere ($x = -45$, $y = -59$, $z = 48$; Z -score = 7.1; $P < 0.05$, corrected for multiple comparisons across the whole brain) and a trend was also evident in the right hemisphere ($x = 56$, $y = -53$, $z = 42$; Z -score = 3.4; $P < 0.001$, uncorrected). Although increased grey-matter density in the inferior parietal cortex was common to both early and late bilinguals, the effect was greater in the early bilinguals in the left ($x = -48$, $y = -62$, $z = 44$; Z -score = 3.5; $P < 0.001$, uncorrected) and right ($x = 45$, $y = -65$, $z = 47$; Z -score = 3.5; $P < 0.001$, uncorrected) hemispheres. No other significant effects were detected in either grey or white matter.

We next investigated whether there was a relation between brain structure and proficiency in the second language and age at acquisition. We tested 22 native Italian speakers who had learned English as a second language at an age between 2 and 34 years. Second-language reading, writing, speech comprehension and production were assessed using a battery of standardized neuropsychological tests (see supplementary information). We found that overall proficiency, as

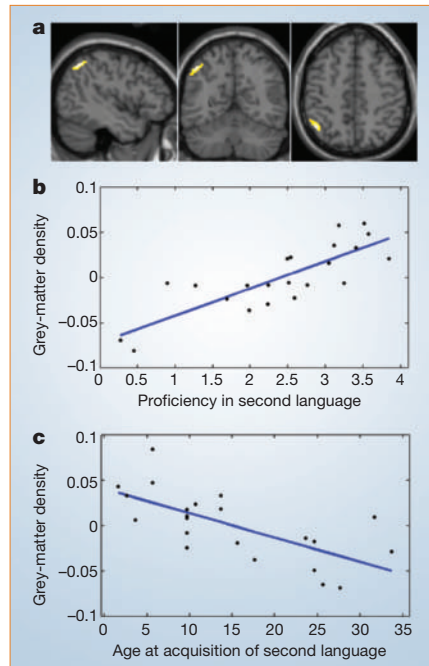


Figure 1 Structural reorganization in the bilingual brain. **a**, Sagittal ($x = -45$), coronal ($y = -59$) and axial ($z = 48$) view of the left inferior parietal region, which has increased grey-matter density in bilinguals relative to monolinguals. **b**, Grey-matter density, measured as cubic millimetres of grey matter per voxel in the left inferior parietal region, as a function of second-language proficiency. Second-language proficiency was estimated for each subject from a battery of standardized neuropsychological tests, using principal component analysis (for details, see supplementary information). **c**, Grey-matter density, measured as for **b**, as a function of age at acquisition.

indexed by principal component analysis, correlated negatively with age at acquisition ($P < 0.01$; $r = -0.855$). Remarkably, VBM revealed that second-language proficiency correlated with grey-matter density in exactly the same left inferior parietal region that we had already identified ($x = -48$, $y = -59$, $z = 46$; Z -score = 4.1; $P < 0.05$, corrected after 10-mm small-volume correction; Fig. 1b). In addition, grey-matter density in this region correlated negatively with the age of acquisition of the second language ($x = -50$, $y = -58$, $z = 42$; Z -score = 3.2; $P < 0.05$, corrected after 10-mm small-volume correction; Fig. 1c). There were no other significant effects in grey or white matter.

We have therefore identified an increase in the density of grey matter in the left inferior parietal cortex of bilinguals relative to monolinguals, which is more pronounced in early rather than late bilinguals, and have also shown that the density in this region increases with second-language proficiency but decreases as the age of acquisition increases.

These effects could result from a genetic

predisposition to increased density, or from a structural reorganization induced by experience⁴. Early bilinguals probably acquire a second language through social experience, rather than as a result of a genetic predisposition. Our findings therefore suggest that the structure of the human brain is altered by the experience of acquiring a second language.

The inferior parietal region that is associated with second-language acquisition corresponds exactly to an area that has been shown by functional imaging to become activated during verbal-fluency tasks^{5,6}. Whether grey-matter reorganization in this region is related to changes in neuropil, neuronal size, dendritic or axonal arborization will be revealed by methods other than whole-brain magnetic resonance imaging.

These results are consistent with growing evidence that the human brain changes structurally in response to environmental demands — for example, structure is already known to alter as a function of learning in domains other than language^{7,8}. We have shown that the degree of this structural reorganization in bilinguals is correlated with their second-language performance. The relationship between grey-matter density and performance discovered here could be an example of a more general structure–function principle that extends beyond the domain of language.

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brief communications arising online

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Mouse transcriptome: Neutral evolution of 'non-coding' complementary DNAs

J. Wang, J. Zhang, H. Zheng, J. Li, D. Liu, H. Li, R. Samudrala, J. Yu, G. K.-S. Wong
(doi:10.1038/nature03016)

Reply: Y. Hyashizaki (doi:10.1038/nature03017)

Mouse transcriptome

Neutral evolution of 'non-coding' complementary DNAs

Arising from: Y. Okazaki *et al. Nature* **420**, 563–573 (2002)

Okazaki *et al.* have argued that as many as 15,815 of 33,409 non-redundant mouse complementary DNAs may represent functional RNA genes¹, on the basis of their findings that some of these cDNAs are confirmed by expressed sequence tagging and are found near CpG islands or polyadenylation signals² — although many are expressed at such low levels that they could not be detected by microarray analysis³. We show here that conservation of these 'non-coding' cDNAs in rats or humans is no better than in an evolutionarily neutral control. Our results indicate that they are either non-functional or, if they are functional, are specific to a given species.

We downloaded FANTOM release 2.0 cDNAs from the authors' website. Table 1 shows the data from the four categories defined by the authors, which we refer to as coding 1 (probably protein), coding 2 (marginal protein), non-coding 1 (marginal RNA), and non-coding 2 (probably RNA). Overall transcript sizes average about 2 kilobases (kb) in each category; most known RNA genes are much smaller than this — for example, the 587 mouse entries in the Rfam database⁴ average 96 base pairs (bp) in length. Larger RNA genes do exist (such as *H19* and *Xist*) and many are stored in the Erdmann database⁵. Another striking difference between the given categories is the increase from 13.4% single-exon genes in coding 1 to 68.7% and 73.1% single-exon genes in non-coding 1 and non-coding 2, respectively.

As an evolutionarily neutral control, we use 'intergenic' sequences of 2 kb in length that are at least 5 kb distant from genes annotated by Ensembl, predicted by FgeneSH, or aligned to cDNAs. Transposons identified by RepeatMasker are excluded, as is the 5% of highly conserved mouse sequence that is under purifying selection⁶. Conversely, we have two positive controls: one is the coding 1 category of protein-coding genes and the other is a set of all known mouse RNA genes. To avoid an overt bias towards small RNA genes, we removed genes smaller than 80 bp in Rfam, leaving behind many encoding splicing factors such as *U1* and *U6*. We then added all the mouse genes in the Erdmann database, which total 40. The resultant set of 321 RNA genes is referred to as 'ncRNAs'.

Genome sequences were taken from the UCSC Genome Browser with time stamp 28 June 2003 (rat) and 10 April 2003 (human). BlastZ (ref. 7) was used for the alignments, with default settings $K=3,000$ and $H=2,200$. The $C=2$ option enabled us to chain exons together. Although the complexities of the chaining procedure may prevent a few multi-exon genes from aligning, this

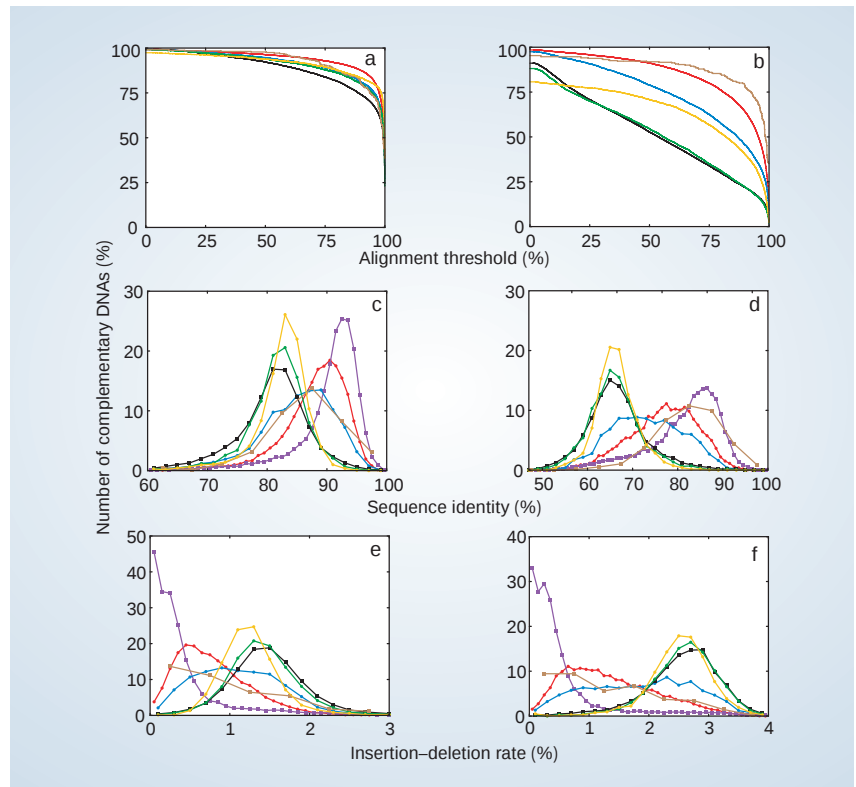


Figure 1 Comparisons between rat (left) and human (right) data. **a, b**, The number of good alignments. **c–f**, Distribution of sequence identities (**c,d**) and insertion-deletion rates (**e,f**) restricted to the good alignments. Each solid dot shows the centre of the bin over which signals were averaged. Red, coding 1; blue, coding 2; black, non-coding 1; green, non-coding 2; brown, ncRNAs; and yellow, intergenic. For panels **c** to **f**, a purple line is added for the CDS region of coding 1.

Table 1 Other attributes of mouse complementary DNAs

	FANTOM categories				Control data sets	
	Coding 1	Coding 2	Non-coding 1	Non-coding 2	ncRNAs	Intergenic
No. of cDNAs	14,317	3,277	11,526	4,280	321	3,450
No. in a single exon	13.4%	35.4%	68.7%	73.1%	90.7%	100%
Size of FL cDNA	2,146 (1,061)	2,174 (1,091)	1,939 (1,019)	1,790 (996)	325 (1,055)	2,000 (0)
Size of 5' UTR	242 (335)	640 (686)	842 (754)	791 (727)	NA	889 (523)
Size of best ORF	1,107 (742)	550 (578)	206 (91)	194 (80)	NA	213 (88)
Size of 3' UTR	836 (746)	983 (807)	891 (770)	805 (718)	NA	898 (524)
BlastX proteins						
$E\text{-value} = 10^{-2}$						
SwissProt	72.4%	55.5%	15.7%	2.4%	0.9%	2.9%
Mouse coding 1	100.0%	59.3%	36.5%	19.0%	4.4%	3.7%
Combined	100.0%	68.0%	37.6%	19.5%	4.4%	4.4%
$E\text{-value} = 10^{-4}$						
SwissProt	68.8%	50.4%	11.1%	0.8%	0.0%	2.0%
Mouse coding 1	100.0%	53.0%	31.0%	12.6%	3.7%	2.5%
Combined	100.0%	62.9%	31.9%	12.8%	3.7%	3.0%
$E\text{-value} = 10^{-6}$						
SwissProt	65.3%	45.5%	6.1%	0.0%	0.0%	1.6%
Mouse coding1	100.0%	47.5%	25.4%	7.7%	2.5%	1.8%
Combined	100.0%	58.2%	26.2%	7.7%	2.5%	2.2%
RepeatMasker	13.7%	27.7%	48.4%	46.4%	3.4%	0.0%

After computing the best open-reading frames (ORFs), left-over flanking sequences are taken to be untranslated regions. Sizes (in base pairs) are described as mean (standard deviation). In the RepeatMasker tallies, we do not count short interspersed elements. UTR, untranslated terminal repeat; NA, not applicable; FL, full-length.

should not be a problem for non-coding cDNAs as most are single-exon. We specified that the fraction of transcript length that is aligned by BlastZ must exceed a predetermined alignment threshold of 25%: this low threshold ensures that our positive controls almost always pass (Fig. 1).

The crucial observation is that the distributions of sequence identity and insertion–deletion ('indel') rate are remarkably similar for non-coding 1, non-coding 2 and intergenic. Even the widths of the distributions, a reflection of the stochastic nature of the underlying evolutionary process, are highly similar. The most well conserved are coding 1 and ncRNAs, and the least well conserved are non-coding 1, non-coding 2 and intergenic. The larger effect is observed in mouse-to-human, because it represents 75 million years of divergence, compared with only 14–24 million years in mouse-to-rat. For the latter comparison, the shift (δ) is small compared with the width (σ); however, it is significant, as it is a shift in an entire distribution, and the oft-cited rule $\delta \gg \sigma$ applies to a point sampled from a distribution.

The simplest explanation is that non-functional transcripts can be produced at low

copy numbers, escape the cell's messenger RNA surveillance system, and yet inflict no damage on the cell. Table 1 highlights two theories. If these are processed pseudogenes, there should be residual similarity to known proteins, especially mouse proteins. Setting to E -values of 10^{-2} , we find that 36.5% and 19.0% of non-coding 1 and non-coding 2 are similar to mouse coding 1. Just 15.7% and 2.4% are similar to SwissProt, because SwissProt does not store translated cDNAs. If random genomic sequence is transcribed, we should find transposon remnants (ignoring short interspersed elements because they are derived from transfer RNAs). This is indeed the case for 48.4% and 46.4% of non-coding 1 and non-coding 2. Note too that the ncRNAs control set is mostly negative for pseudogenes and random genomic sequence.

Given that all of the best techniques for detecting RNA genes depend on sequence conservation^{8,9}, the absence of this cannot be summarily dismissed, even if isolated examples of RNA genes being weakly conserved can be found¹⁰. Extraordinary claims require extraordinary proof — this is particularly true when much of the data support an alternative interpretation that they are

simply non-functional cDNAs.

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Okazaki et al. reply — Wang *et al.*¹ challenge our suggestion that almost half of the 33,407 representative putative full-length cDNAs identified by RIKEN are probably non-coding RNAs². The challenge is based on bioinformatic analyses showing that the level of conservation of these sequences is no greater than that observed for intergenic sequences, and less than that observed in a control set of documented ncRNAs.

The analyses of Wang *et al.* have several problems. Their positive control set is heavily biased towards structural and catalytic RNAs. It contains only 19 non-redundant regulatory ncRNAs from the Erdmann database³, with the majority being drawn from the Rfam RNA database⁴, which — despite the 80-nucleotide cutoff used to remove very small RNAs — contains mainly infrastructural RNAs, such as spliceosomal and small nucleolar RNAs, many of which are covariance-model structure-based predictions from large genomic data sets. The large dichotomy in the control set is shown by their average size of 325 nucleotides, with standard deviation of 1,055 nucleotides¹.

There is ample evidence that known regulatory ncRNAs are, in the main, much less conserved than protein-coding sequences. For example, the 110-kb murine ncRNA Air, which is involved in imprinting of the *Igf2r* locus (and is represented in the RIKEN database), has no equivalent transcript in humans⁵, and shows no significant homology

with either the human or rat genomes. The rat ncRNA Bsr does not occur in human or mouse. The human ncRNA DISC2 is not conserved in mouse or Fugu, despite the fact that its surrounding transcripts (including its putative antisense target DISC1) are conserved across mammals and Fugu⁶. Xist shows low homology (60%) between mammalian species, despite its identical function in X-chromosome inactivation⁷. The antisense transcript Tsix, also important to this process, is poorly conserved between species⁷. Indeed, alignment of known regulatory ncRNAs among mammalian species shows that there is large divergence in the percentage of alignable sequences and that their overall homology is low and indistinguishable from intergenic sequences.

The validity of 'intergenic' sequences as a negative control is also questionable. Although it is thought that only 5% of the mammalian genome is under purifying selection, this figure was obtained from comparison of the mouse and human genomes, compared with sequences assumed to be evolving neutrally⁸. However, multivariate analyses of the *CFTR* (ref. 9) and *SIM2* (ref. 10) loci showed that intergenic and intronic sequences exhibit patterns of conservation that are not evident from pairwise comparisons alone. That is, the sequences exhibiting conservation depend on which species are being compared, implying that more of the genome is under evolutionary

selection (both positive and negative) than has been appreciated, with many non-coding sequences, presumably regulatory, being differently conserved in lineage-specific ways.

The possible sources of contamination of cDNA libraries are genomic and pre-mRNA sequences, and 'transcriptional noise'. Our analyses have shown that there is insignificant genomic contamination in the RNA preparations used to construct cDNA libraries, and also that most putative ncRNA sequences are not derived from introns of protein-coding genes. The concept of transcriptional noise derives from studies of stochastic transcription¹¹, but this does not mean that such transcription occurs from illegitimate promoters, nor is there any evidence for this. Our published¹² and unpublished analyses show that many of the putative ncRNA transcripts exhibit both tissue-specific and dynamic regulation of their expression in relation to external cues. Our findings agree with independent analyses using genomic arrays, which conclude that the human genome contains comparable numbers of protein-coding and non-coding genes that are under the control of common transcription factors and environmental signals^{13,14}. They also agree with molecular genetic analyses of well studied loci, which invariably show that at least half of documented transcripts are non-coding¹⁵.

(This response was prepared with input from Shintaro Katayama, Harukazu Suzuki,

Ken Pang and John Mattick.)

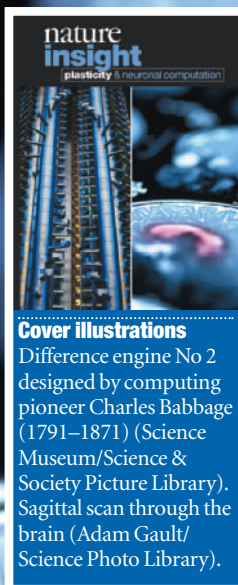
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nature insight

plasticity & neuronal computation



Cover illustrations

Difference engine No 2 designed by computing pioneer Charles Babbage (1791–1871) (Science Museum/Science & Society Picture Library). Sagittal scan through the brain (Adam Gault/Science Photo Library).

If you read these words from Marvin Minsky: “minds are what brains do” and “doing means changing”, your brain’s fine structure may be durably altered. Such is neuronal plasticity, a concept that has found a home in many areas of neuroscience, from brain repair to learning and memory. But plasticity is not only a reaction to change; it is also a source of change. This Insight considers plasticity as the critical engine of neuronal computation.

Purely elastic systems cannot compute much: imagine an abacus with springs between the beads. But assemble the simplest storing or switching devices, such as Charles Babbage’s mechanical gears or silicon-based flip-flops, and you get a universal computer. Living organisms, from bacteria to elephant, are packed with comparable switches, gates and stores. From protein allostery and trafficking to long-range neuromodulation, everything biological produces adaptive computation.

Synapses, for example, change strength in real time, as Bernard Katz observed fifty years ago — not just slowly to sustain learning and memory. And there is a growing appreciation of how much they differ from passive linear junctions. Short-term plasticity allows synapses to decode spike trains, transmitting some and blocking others. And because synapses have distinct histories, a neuron has not one but myriads of outputs, so temporal codes translate into spatial maps.

Therefore, plasticity emerges as perhaps the deepest and most pervasive source of computational power in the brain. The following reviews illustrate this idea from millisecond computations in synapses to life-long information storage in cortex, and from digestive control in crabs to general intelligence and value judgement in humans. Each piece brings together computer and bench neuroscientists to offer a fresh meeting of experiment and theory.

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Tanguy Chouard Senior Editor

review articles

760 Computational roles for dopamine in behavioural control

P. R. Montague,
S. E. Hyman
& J. D. Cohen

768 Generalization in vision and motor control

T. Poggio & E. Bizzi

775 Neural networks and perceptual learning

M. Tsodyks & C. Gilbert

782 Cortical rewiring and information storage

D. B. Chklovskii,
B. W. Mel & K. Svoboda

789 Plasticity in single neuron and circuit computations

A. Destexhe & E. Marder

796 Synaptic computation

L. F. Abbott
& W. G. Regehr

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Computational roles for dopamine in behavioural control

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Neuromodulators such as dopamine have a central role in cognitive disorders. In the past decade, biological findings on dopamine function have been infused with concepts taken from computational theories of reinforcement learning. These more abstract approaches have now been applied to describe the biological algorithms at play in our brains when we form value judgements and make choices. The application of such quantitative models has opened up new fields, ripe for attack by young synthesizers and theoreticians.

The concept of behavioural control is intimately tied to the valuation of resources and choices. For example, a creature that moves left instead of right may forgoe the food and other resources that it could have obtained had it chosen right.

Such stark, yet simple economic realities select for creatures that evaluate the world quickly and choose appropriate behaviour based on those valuations. From the point of view of selection, the most effective valuations are those that improve reproductive success. This prescription for valuation yields a formula for desires or goals: an organism should desire those things deemed most valuable to it. All mobile organisms possess such discriminatory capacities and can rank numerous dimensions in their world along axes that extend from good to bad. A kind of facile biological wisdom is built into these simple observations and we should expect valuation mechanisms to be built into our nervous systems at every level, from the single neuron to the decision algorithms used in complex social settings.

These ideas have recently been upgraded from provocative biological musings to real computational models of how the nervous system sets goals, computes values of particular resources or options, and uses both to guide sequences of behavioural choices. Such models have cast as important players our midbrain's dopamine neurons, whose actions define 'rewards' — our goals or desires — that should be sought. These neurons have a central role in guiding our behaviour and thoughts. They are hijacked by every addictive drug; they malfunction in mental illness; and they are lost in dramatically impairing illnesses such as Parkinson's disease. If dopamine systems are overstimulated, we may hear voices, experience elaborate bizarre cognitive distortions, or engage excessively in dangerous goal-directed behaviour. Dopamine function is also central to the way that we value our world, including the way that we value money and other human beings.

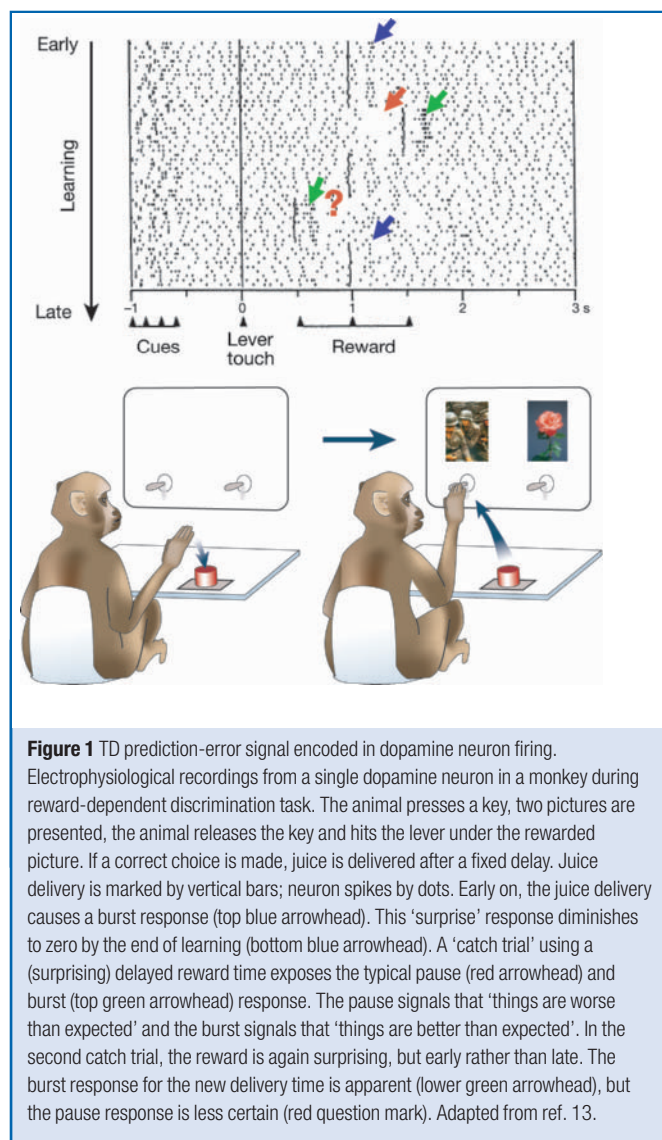
The full story of behavioural control requires vastly more than simple models of dopaminergic function. But here we show how one branch of computational theory — reinforcement learning — has informed both the design and interpretation of experiments that probe how the dopamine system influences sequences of choices made about rewards. These models are maturing rapidly and may even guide our understanding of other neuromodulatory systems in the brain, although such applications are still in their infancy.

Reinforcement signals define an agent's goals

Reinforcement learning theories seek to explain how organisms learn to organize their behaviour under the influence of rewards¹. 'Reward' is an old psychological term defined by Merriam Webster's dictionary as "a stimulus administered to an organism following a correct or desired response that increases the probability of occurrence of the response". Here, we show that current theories of reinforcement learning provide a formal framework for connecting the physiological actions of specific neuromodulatory systems to behavioural control. We focus on dopaminergic systems primarily because they have been most extensively modelled and because they play a major role in decision-making, motor output, executive control and reward-dependent learning^{2–5}. We show how the dopaminergic models provide a way to understand neuroimaging experiments on reward expectancy and cognitive control in human subjects. Finally, we suggest that this same class of model has matured sufficiently for it to be used to address important disturbances in neuromodulation associated with many psychiatric disorders.

Despite its name, reinforcement learning is not simply a modern recapitulation of stimulus–response learning, familiar from the classical and instrumental conditioning literature⁶. Traditional stimulus–response models focused on how direct associations can be learned between stimuli and responses, overlooking the possibility that numerous internal states intervene between the stimulus and its associated response. However, animals clearly have covert internal states that affect overt, measurable behaviour. Reinforcement learning theory explicitly models such intervening states, assumes that some are more desirable than others, and asks how do animals learn to achieve desired states and avoid undesirable ones as efficiently as possible? The answer to this question shows how reinforcement signals define an agent's goals. For simplicity, we focus only on rewards. However, the same story can be told using negative reinforcers (punishments).

We refer to the state engendered by a reward as a 'goal'. Goals can exist at numerous levels and direct behaviour over many timescales. Goals for humans range from the most basic (for example, procuring something to eat in the next minute) to the most abstract and complex (such as planning a career). In reinforcement learning, it is assumed that the fundamental goal of the agent (learner) is to learn to take actions that are most likely to lead to the greatest accrual of rewards in the future. This goal is achieved under the guidance of simple scalar quantities called reinforcement signals. These signals



serve to criticize specific actions or contemplated actions with respect to how effectively they serve the agent's goals. In reinforcement learning, one common goal is the maximization of total future reward⁶.

Every reinforcement learning system possesses three explicitly implemented components: (1) a 'reinforcement signal' that assigns a numerical quantity to every state of the agent. Reinforcement signals can be negative or positive. They define the agent's immediate goals by reporting on what is good or bad 'right now'; (2) a stored 'value function' that formalizes the idea of longer-term judgments by assigning a 'value' to the current state of the agent (see Box 1); (3) a 'policy function' that maps the agent's states to its actions. Policies are typically stochastic: they assign a probability to each possible action that can be taken from the current state, with the probability weighted by the value of the next state produced by that action.

A more concrete description reads as iterations of the following recipe: (1) organism is in state X and receives reward information; (2) organism queries stored value of state X; (3) organism updates stored value of state X based on current reward information; (4) organism selects action based on stored policy; and (5) organism transitions to state Y and receives reward information.

In one form of reinforcement learning called temporal-difference learning, a critical signal is the reward-prediction error (also called the temporal-difference, or TD error)⁷⁻⁹. Unlike the well-known psychological learning rule proposed by Rescorla and Wagner¹⁰ in 1972, this error function is not simply a difference between the received

reward and predicted reward; instead, it incorporates information about the next prediction made by the reward-prediction system¹¹. In words: current TD error = current reward + γ next prediction - current prediction. Here, the words 'current' and 'next' refer respectively to the present state and to the subsequent state of the learner; γ is a factor between 0 and 1 that weights the relative influence of the next prediction. By using this reward-prediction error to refine predictions of reward for each state, the system can improve its estimation of the value of each state, and improve its policy function's ability to choose actions that lead to more reward.

The reward-prediction-error hypothesis

Over the past decade, experimental work by Wolfram Schultz and colleagues has shown that dopaminergic neurons of the ventral tegmental area and substantia nigra show phasic changes in spike activity that correlate with the history of reward delivery¹²⁻¹⁶. It was proposed that these phasic activity changes encode a 'prediction error about summed future reward' (as described above): this hypothesis has been tested successfully against a range of physiological data²⁻³. The 'pause' and 'burst' responses of dopamine neurons that support a reward-prediction-error hypothesis are shown in Fig. 1. The bursts signal a positive reward-prediction error ('things are better than expected'), and the pauses signal a negative prediction error ('things are worse than expected'). Activity that remains close to the baseline signals that 'things are just as expected'. However, this verbal interpretation of dopaminergic activity belies the sophistication of the underlying neural computations¹ (Box 1).

Value binding and incentive salience

We have presented theoretical evidence that phasic bursts and pauses in midbrain dopaminergic activity are consistent with the formal construct of a reward-prediction error used by reinforcement learning systems (Fig. 1; Box 1). This interpretation is consistent with a long history of physiological and pharmacological data showing that dopamine is involved in appetitive approach behaviour¹⁷⁻¹⁹, and is a key component in the pathologies of behavioural control associated with drug addiction²⁰⁻²¹.

One finding offered as a challenge to the models discussed so far is that antagonism of dopamine receptors does not change the appetitive value of food rewards but does prevent the treated animal from initiating actions that allow it to obtain the food reward^{17,22}. In these experiments, animals treated with dopamine-receptor blockers are virtually unable to link sequences of actions to obtain a food reward, but they will consume the same amount as untreated animals if they are moved close to the food rewards by the experimenter (Fig. 2). This conclusion also holds for the inhibition of dopamine neuron firing by gamma-aminobutyric acid (GABA) injected directly into the ventral tegmental area (Fig. 2). These data suggest that interfering with dopamine transmission does not alter the internal evaluation of rewards, but simply the ability to act on those valuations. Addressing these data at a conceptual level, Berridge and Robinson have proposed that dopamine mediates the 'binding' between the hedonic evaluation of stimuli and the assignment of these values to objects or acts¹⁷. They call this idea 'incentive salience'. Although competing psychological explanations differ with respect to the specific claims of incentive salience^{19,23,24}, they all agree that dopamine release and binding is a necessary link between the evaluation of potential future rewards and the policy (sequence of actions) that acquires the rewards. Here, we refer to this link as value binding and distinguish three components: (1) the value computation; (2) the link to a policy (value binding); and (3) execution of the policy.

Incentive salience and actor-critic models

There is a class of reinforcement learning model, called the actor-critic that is closely related to the Berridge and Robinson model for the role of dopamine in value and action learning^{1,9}. In these models, the 'critic' carries the reward-prediction error associated

Box 1

Value functions and prediction errors

The value function

In the simplest TD models of dopamine systems, the reward-prediction error depends on a value function that equates the value V of the current state s at time t with the average sum of future rewards received up until the end of a learning trial.

$$\begin{aligned} V(s_t) &= \text{average sum of future rewards delivered from state } s_t \text{ until} \\ &\quad \text{the end of a learning trial} \\ &= \text{average } [r_t + r_{t+1} + r_{t+2} + \dots + r \text{ (trial's end)}] \\ &= E \left[\sum_{\tau \in \text{Trial}} r(\tau) \right] \end{aligned} \quad (1)$$

E is the expected value operator. There are two sources of randomness over which the above averaging occurs. First, the rewards in a trial $[r_t + r_{t+1} + r_{t+2} + \dots + r \text{ (trial's end)}]$ are random variables indexed by the time t . For example, r_{t+2} is a sample of the distribution of rewards received two timesteps into the trial. The idea is that the animal can learn the average value of these rewards by repeating learning trials, and by revisiting state s_t sufficiently frequently for its nervous system to be able to estimate the average value of each of the rewards received from state s_t until the end of the trial. The second source of randomness is the probabilistic transition from one state at time t to a succeeding state s_{t+1} at a later time $t+1$. The value function, stored within the nervous system of the creature, provides an assessment of the likely future rewards for each state of the creature; that is, the value must somehow be associated with the state. However, as written in equation (1), it would be virtually impossible to make good estimates of the ideal $V(s_t)$ as it is now defined. This is because the creature would have to wait until all rewards were received within a trial before deciding on the value of its state at the beginning of the trial. By that time, it is too late for such a computation to be useful. This problem becomes worse in real-world settings. Fortunately, equation (1) provides a way out of this dilemma because it obeys a recursion relation through time:

$$V(s_t) = E[r_t] + V(s_{t+1}) \quad (2)$$

This recursion relation shows that information about the value of a state s_t is available using only the value $V(s_{t+1})$ of the current state s_t and

the value of its successor state s_{t+1} . Until this point, we have been discussing the ideal case for V . However, as indicated above, V cannot be known exactly in the real world. Instead, an estimate $\hat{V}$ of V must be formed within the nervous system. The TD algorithm learns an approximation $\hat{V}$ of the value function V . It uses a prediction-error signal:

$$\begin{aligned} \delta(t) &= \text{prediction error (t)} = E[r_t] + \hat{V}(s_{t+1}) - \hat{V}(s_t) \\ &\approx \text{current reward} + \text{next prediction} - \text{current prediction} \end{aligned} \quad (3)$$

This TD error signal reproduces the phasic burst and pause responses measured in dopamine neurons recorded in alert monkeys during learning tasks. The next value of each adaptable weight $w(t+1)$ used to estimate V is incremented or decremented in proportion to the product of the current prediction error $\delta(t)$ and the current representation $s(t)$ of the stimulus responsible for the prediction.

$$w(t+1) = w(t) + \lambda s(t) \cdot \delta(t) \quad (4)$$

Here, λ is a learning rate.

Exponential discounting of future rewards

The artificial truncation at the end of a trial (equation (1)) can be handled theoretically in several ways. One popular formalization is to weight the near future more than the distant future. In this case, the analogue to equation (1) takes the form:

$$\begin{aligned} V_d(\delta(t)) &= \text{average sum of discounted future rewards} \\ &= \text{average } [\gamma^0 r(t) + \gamma^1 r(t+1) + \gamma^2 r(t+2) + \dots] \text{ for } 0 < \gamma < 1 \\ &= E \left[\sum_{\tau \geq t} \gamma^{\tau-t} r(\tau) \right] \end{aligned}$$

Using this weighted version of the value function, the learning episodes for a creature do not have to be artificially divided into 'trials'. An analogous reward-prediction-error signal can be formed and used in the same manner as above:

$$\begin{aligned} \delta(t) &= \text{prediction error (t)} = E[r_t] + \gamma \hat{V}(s_{t+1}) - \hat{V}(s_t) \\ &\approx \text{current reward} + \gamma \cdot \text{next prediction} - \text{current prediction} \end{aligned} \quad (5)$$

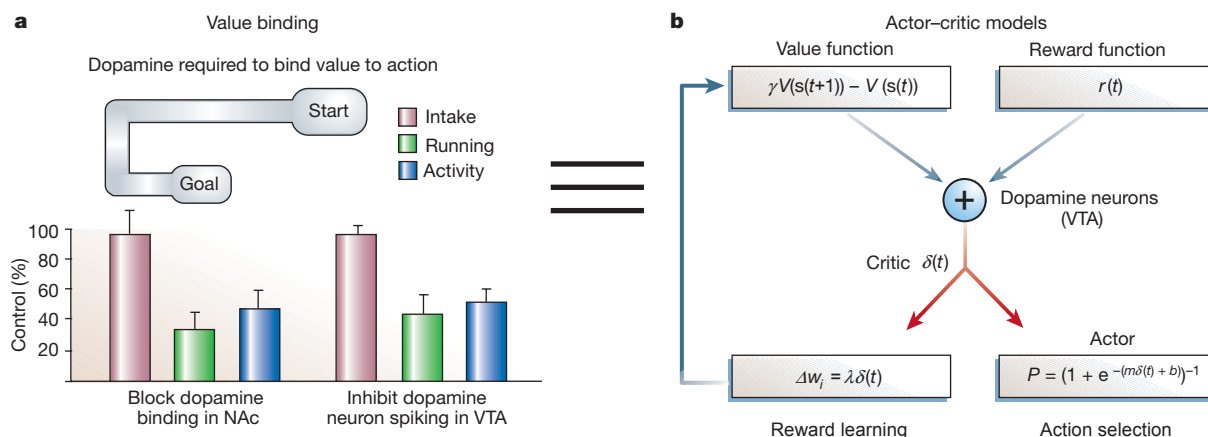


Figure 2 Equating incentive salience with the actor-critic model. **a**, Rats are trained to run a maze to acquire sugary water. If dopaminergic spiking is blocked (left histograms) in the VTA, then rats will generally not run down the maze to get a reward and are less active. However, if the experimenter moves them to the sugary water, the rats drink exactly the same amount as untreated rats. This suggests that the (hedonic) value of the sugary water has been computed but that the capacity to

bind this value to actions required to obtain the water fails to function. The same effect results if dopamine's interaction with its receptor is blocked in an important downstream target of dopamine projections (right histograms). Adapted from refs 22 and 25. **b**, Actor-critic models use dopamine-encoded prediction-error signal in two roles: (1) to learn stimulus-reward associations, and (2) to assess actions or contemplated actions (notations are as in Box 1). Adapted from refs 2, 25, 83.

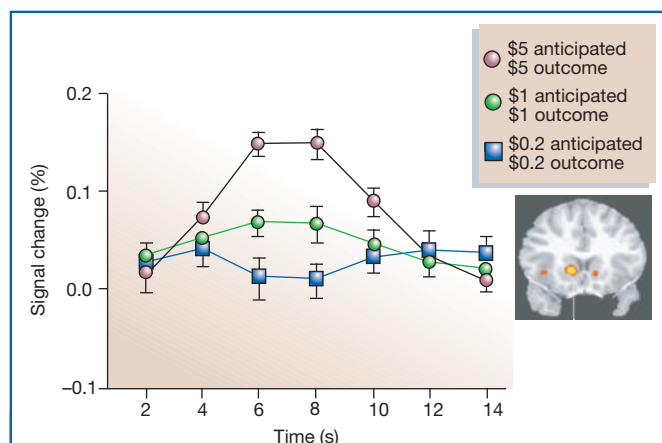


Figure 3 Scaled responses to a monetary reward in the ventral striatum. Action is required to receive a reward. The haemodynamic response is modulated by the amount of money received. In both cases, positive deviations in expectations make the responses bigger. Adapted from ref. 38.

with the states of the organism. The ‘actor’ uses this signal, or a closely related one, to learn stimulus–action associations, so that actions associated with higher rewards are more likely to be chosen. Together, these two components capture many features of the way that animals learn basic contingencies between their actions and the rewards associated with those actions. The original hypothesis concerning the role of dopamine in reinforcement learning proposed just such a dual use of the reward–prediction–error signal^{2,25}. McClure and colleagues recently extended this original learning hypothesis to address the Berridge and Robinson model²⁶. This work suggests a formal relationship between the incentive-salience ideas of Berridge and Robinson and actor–critic models in which incentive salience is equivalent to the idea of expected future value formalized in reinforcement learning models (Fig. 2).

Actor–critic models are now being used to address detailed issues concerning stimulus–action learning⁸. For example, extensions to actor–critic models have addressed the difference between learning goal-directed approach behaviour and learning automatic actions (habits), such as licking. There are several behavioural settings that support the contention that habit learning is handled by different neural systems from those involved in goal-directed learning^{27,28}. Dayan and Balleine have recently offered a computational extension to actor–critic models to take account of this fact²⁹.

Rewards, critics and actors in the human brain

Recent functional magnetic resonance imaging (fMRI) experiments have used reward expectancy and conditioning tasks to identify brain responses that correlate directly with rewards, reward–prediction–error signals (critic), and signals related to reward-dependent actions (actor). Many of these experiments have used reinforcement learning models as a way to understand the resulting brain responses, to choose design details of the experiment, or to locate brain responses associated with specific model components^{30–34}.

Human reward responses

Responses to rewarding stimuli have been observed consistently from the same set of subcortical regions in human brains, suggesting that neurons in these regions respond to a wide spectrum of triggers. In a series of elegant papers, Breiter and colleagues used fMRI to record brain responses to beautiful visual images³⁵ and drugs that induce euphoria (cocaine)³⁶. The brain structures they identified included the orbitofrontal cortex (OFC), amygdala (Amyg), nucleus accumbens (NAc; part of the ventral striatum), sublentiform extended amygdala (SLEA; part of the basal forebrain), ventral

tegmental area (VTA), and hypothalamus (Hyp). All these regions have topographically organized reciprocal connections with the VTA — one of the primary dopaminergic nuclei in the brainstem.

Particularly strong reward responses have been observed in the ventral striatum where numerous studies have shown that even abstract proxies for reward (money) cause activations that scale in proportion to reward amount or deviation from an expected payoff^{37–39}. Similar results have been found by a variety of groups using both passive and active games with monetary payoffs^{40–42}. A prominent activation response to monetary payoff was observed by Knutson and colleagues in the NAc and is shown in Fig. 3. The NAc, like the OFC and other parts of the prefrontal cortex (PFC), is densely innervated by dopaminergic fibres originating from neurons housed in the mid-brain. Other work has shown that simply changing the predictability of a stimulus will activate the NAc and surrounding structures in the ventral parts of the striatum³⁰. The picture emerging from this work is that responses in this region may reflect an encoding of rewards along a common valuation scale⁴³.

Human critic responses

One of the most important contributions of reinforcement learning theory has been to distinguish between the signalling of the reward itself, and the computation of the reward–prediction error. Using passive tasks with a juice reward, reward–prediction errors have been shown to activate structures in the ventral striatum^{30,44}. Recently, two independent groups used passive learning paradigms to visualize reward–prediction–error signals in overlapping regions of the ventral putamen^{32,33} (Fig. 4). The cingulate cortex is another area that has been associated with reinforcement learning signals that seem to be reward–prediction errors. The error-related negativity (ERN) is a scalp-recorded event-related potential (ERP), believed to originate from the anterior cingulate cortex, that is consistently observed about 100 msec following the commission of an error^{45,46}. Similar potentials have been observed following negative feedback or unexpected losses in gambling tasks^{47–49}. Holroyd and Coles have proposed that these potentials reflect a negative reward–prediction–error signal, and this idea has been tested under a variety of conditions^{50–52}. Recently, fMRI evidence has suggested that a region of anterior cingulate cortex responds under many of the same conditions as the ERN: activity is affected by both errors and negative feedback⁵³.

Human actor responses

One implication of reinforcement theory for behaviour concerns the relationship between reward–prediction errors (critic signals) and action selection (actor signals). As discussed in Box 1, the critic signal can be used for reward learning and to adjust the future selection of reward–yielding actions. Success in the use of fMRI to detect reward–prediction–error signals inspired O’Doherty and colleagues to carry out a clever, but simple experiment designed to relate critic signals to action selection³⁴. The experiment used a conditioning paradigm that was carried out in two modes. The first required an action to obtain a juice reward and the second did not. This experiment showed that activity in the dorsal striatum correlated with the prediction–error signal only when an action was needed to acquire the juice reward (Fig. 4c). There was no similar activity in this area when the juice was passively delivered. This finding is important because the dorsal striatum is involved in the selection and sequencing of actions.

Neuromodulation and cognitive control

Our consideration of reinforcement learning theory so far has focused on simple situations, involving the association of a stimulus with a reward, or with the selection of an action that leads to an immediate reward. In the real world, however, accrual of reward may require an extended sequence of actions. Furthermore, we have considered only a highly abstracted definition of the goal of the organism — the maximization of cumulative future rewards. However, many different forms of reward (and associated actions) may be

valued by an organism (for example, the procurement of nutrition, provision of safety, reproduction). This suggests that the construct of a goal needs to be refined to describe the variety of goal-directed behaviour in which humans engage. The guidance of behaviour in the service of internally represented goals or intentions, is often referred to as the capacity for cognitive control. Recent theories of cognitive control have elaborated on basic reinforcement learning mechanisms to develop models that specifically address the two challenges suggested above: (1) the need to learn and control sequences of actions required to achieve a goal; and (2) the need to represent the variety of goals that an organism may value. Here, we focus on the first of these challenges, but see refs 54 and 55 for a discussion of the latter.

Prefrontal goals

Pursuit of a goal (for example, going to the car, driving to the grocery store, or locating the refrigerated section to buy milk), can often

require an extended sequence of actions. Theories of cognitive control consistently implicate the PFC as a site where representations of goals are actively maintained and used to select goal-directed behaviours⁵⁴. The involvement of the PFC is motivated by three diverse classes of observations: (1) the PFC can support sustained activity in the face of distracting information^{56,57}; (2) damage to the PFC produces deficits in goal-directed behaviour^{58,59}; and (3) the PFC is selectively engaged by tasks that rely heavily on the active representation of goal information⁶⁰.

Dopamine gating hypothesis

One problem with the simple hypothesis that the PFC actively maintains goal representations is that this does not indicate how or when this information should be updated. Failure to appropriately update goal representations will lead to perseverative behaviour, whereas failure to adequately maintain them will result in distractibility. Indeed, disturbances of the PFC are known to be associated with distractibility, perseveration, or both⁶¹. What is required is a mechanism that can signal when the goal representation should be updated. Recently, it has been proposed that dopaminergic signals from the VTA implement this mechanism, by controlling the 'gating' of afferent information into the PFC^{55,62} (Fig. 5). According to this gating hypothesis, the PFC is resistant to the influence of afferent signals in the absence of phasic dopamine release, allowing it to preserve the currently maintained goal representation against impinging sources of interference. However, stimuli that signal the need to update the goal representation elicit a phasic dopamine response that 'opens the gate' and allows afferent signals to establish a new goal representation in the PFC.

Reinforcement learning and working memory

How does the dopamine system know which stimuli should elicit a gating signal and which should not? One plausible answer to this question comes directly from the reinforcement learning theory of dopamine function. A gating signal is required to update the PFC when a stimulus occurs in the environment which indicates that a more valuable goal can be achieved if behaviour is redirected towards that goal (for example, a light signalling that a reward can be acquired by going to some new location). In reinforcement learning terms, this corresponds to a positive reward-prediction error: the value of the current state is better than expected. According to the reinforcement learning theory of dopamine function, this is associated with a phasic burst in dopamine activity. In other words, reinforcement learning theory predicts that phasic dopamine responses will occur precisely when needed to produce a gating signal. Furthermore, insofar as the phasic dopamine response acts as a learning signal, it will strengthen the association of the current predictor, for example, the light, with the goal representation in the PFC. It will also strengthen the tendency of the light to elicit a phasic dopamine response when it recurs in the future. The learning here is analogous to the simple 'light-predicts-juice' experiments described earlier, except that now 'light predicts goal representation in the PFC', which in turn leads to the accrual of reward. This proposal shows how a prefrontal representation that plays a causal role in the acquisition of some later reward comes to be selected and reinforced.

Assuming that dopamine generates both learning and gating effects, the dopamine system provides a mechanism for learning which stimuli should elicit a gating signal to update goal representations in the PFC. Consistent with this hypothesis, the parameter used to implement the learning effects of dopamine in formal models of reinforcement learning^{2,8,30,63} bears a remarkable similarity to the parameter used to implement gating effects in models of dopamine-based gating signals in the PFC⁶³. Recent computational modelling work has demonstrated that implementing concurrent effects of dopamine phasic signals on reinforcement learning and gating allows a system to associate stimuli with the gating signals that predict reward, and so learn how to update representations appropriately in the PFC^{62,64,65}.

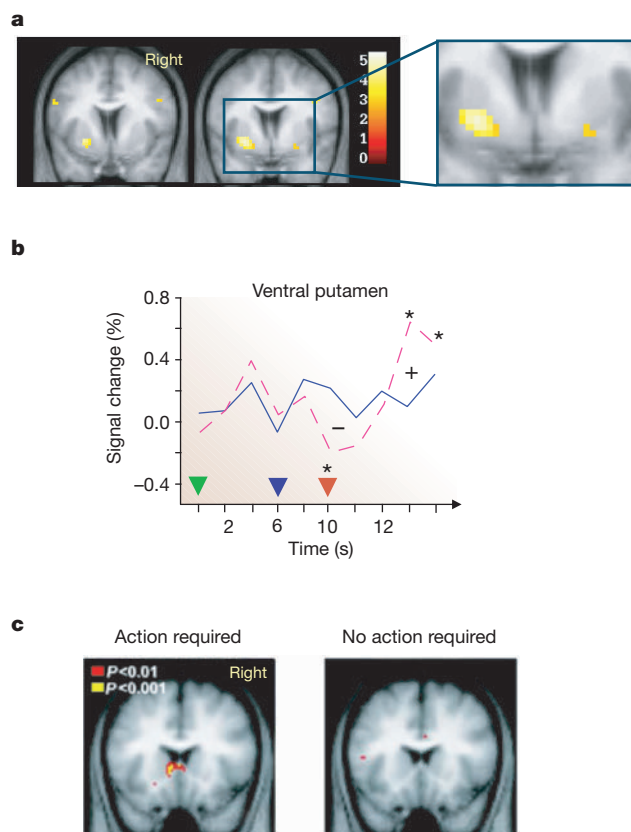


Figure 4 Detecting actor and critic signals in the human brain using fMRI. **a**, A simple conditioning task reveals a TD-like prediction-error response (critic signal) in the human brain. A cue is followed by the passive delivery of pleasant-tasting juice while subjects are scanned. The highlighted activation is located in the ventral part of the striatum (the putamen) — a region known to respond to a range of rewards. The activation represents the brain response that correlates with a continuous TD-like error signal. Adapted from ref. 30. **b**, A similar experimental design, but in this case a single prediction error of each polarity (positive and negative) can be seen in the ventral putamen during a surprising catch trial. Predictive sensory cue (green arrowhead); normal reward-delivery time (blue arrowhead); delayed reward time on catch trials (red arrowhead). Average BOLD (blood oxygenation level dependent) response in normal trials (solid line) and delay trials (dashed line). Adapted from ref. 32. **c**, Identification of actor response in dorsal striatum. A conditioning task is carried out in two modes requiring: (1) a button press (an action); and (2) no action at all. The dorsal striatum — a region involved in action selection — responds only during the mode where action is required and shows no response when action is not required. This is the first demonstration of an actor response detected in the human brain. Adapted from ref. 33.

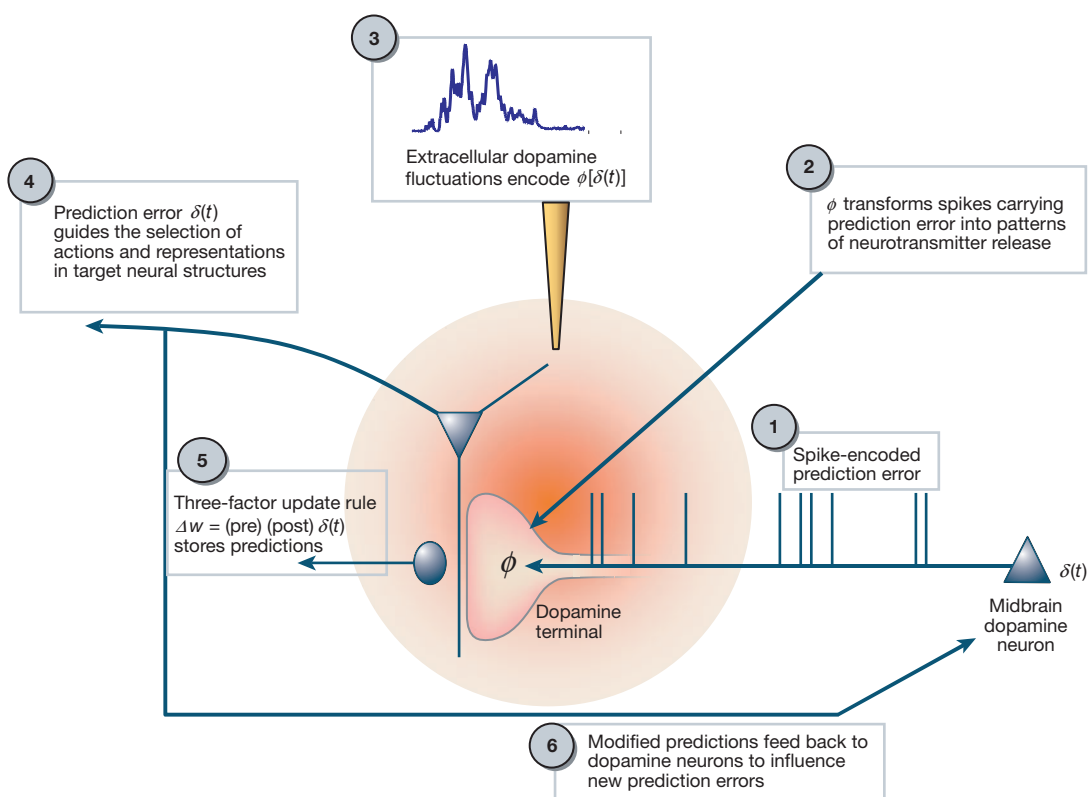


Figure 5 The flow and transformation of signals carried by the dopaminergic system. This system is now thought to be one part of a large, sophisticated neural system for valuation. (1) Dopamine neurons encode reward-prediction-error signals as modulations in their baseline firing rate; (2) transformation ϕ characterizes the way in which modulation of firing rate changes dopamine delivery (ϕ is known to be non-linear)⁷²; (3) movement of dopamine through the extracellular space carries prediction-error information away from the synapse;

(4) dopamine delivery to target structures controls a range of functions including the gating of working memory and the selection of specific actions; (5) any multiplicative learning rule that depends on the dopamine-encoded prediction error is able to store predictions, a vast improvement over simple storage of correlations familiar from hebbian learning; (6) changes in target structures act to adjust predictions, which are delivered back to dopamine neurons through long-range connections.

Recent work has begun to explore the hypothesis that the basal ganglia provide a mechanism for selective updating of goal representations within the PFC. This proposes that an important component of dopaminergic gating takes place in the basal ganglia, acting selectively on recurrent pathways that run from the PFC through the basal ganglia and back to the PFC. Computational models of the basal ganglia have shown how this system can learn tasks that require hierarchical updating of goal representations.

Neuromodulation and pathologies of cognitive control

Reinforcement learning theory provides a formal framework within which to explore quantitatively the effects that alterations in dopamine function may have on behaviour. We consider here two disorders in which it has long been recognized that dopamine plays a major role: drug addiction and schizophrenia.

Disturbances of dopamine in addiction

Perhaps the best understood pathology of dopamine excess is drug addiction, which is defined as compulsive drug use despite serious negative consequences. Once a pattern of compulsion is established, it often proves remarkably persistent. Even when addicted individuals have been drug-free for extended periods, drug-associated cues can readily lead to relapse. Addictive drugs such as cocaine, amphetamine and heroin all increase dopamine concentrations in the NAc and other forebrain structures by diverse mechanisms^{20,66} and are highly reinforcing.

A new way to conceptualize the process of addiction is in the terms described above^{21,67}. If dopamine plays a central role in both

stimulus–reward learning and stimulus–action learning, and addictive drugs result in greater and longer-lasting synaptic dopamine concentrations than any natural reward, several predictions follow. Cues that predict drug availability would take on enormous incentive salience, by means of dopamine actions in the NAc and PFC, and complex drug-seeking behavioural repertoires would be powerfully consolidated by dopamine actions in the dorsal striatum²¹. In addition, dopamine effects in the PFC may impair the ability of the addicted person to suppress prepotent drug-seeking behaviour¹⁷. Given that certain risk-associated behaviour produces phasic dopamine release, and given the similarities between the development of drug addiction and pathologic gambling, it is interesting that early human neuroimaging results suggest that similar brain circuits may be involved⁶⁸.

Collectively, these results point to a hijacking of dopamine signals in PFC and limbic structures by addictive drugs. Because these drugs directly engage dopamine-mediated reinforcement learning signals, they generate a feedback loop that reinforces behaviour leading to drug consumption, establishing a vicious cycle of action and learning that explains the compulsive nature of drug addiction. The degree to which these drugs disrupt both phasic and tonic dopamine signals is not yet clear. However, the reinforcement learning models described above provide a framework for considering possible effects. For the learning effects, over-training with cues that predict drug delivery is a natural consequence of the role of phasic dopamine in learning. The PFC gating signal would also be unnaturally disrupted by selecting and over-learning grossly maladaptive prefrontal representations. These two effects would conspire to yield a representation of the world that is grossly biased towards drug-related cues. In addition,

repeated selection of maladaptive prefrontal representations would catastrophically rearrange the way in which normal functions were categorized within the PFC. In this framework, the addicted person's PFC can no longer even categorize decision problems correctly, much less regain control over the choices that their nervous systems deem valuable. The advantage now is that the reinforcement learning models provide a parameterized view of these problems and may well yield new directions in future work.

Disturbances of dopamine in schizophrenia

Disturbances of dopamine function are also known to have a central role in schizophrenia. This was first suggested by the discovery of the neuroleptic drugs that are effective in ameliorating the hallucinations and delusions associated with this illness. The clinical efficacy of these drugs correlates directly with their potency in blocking dopaminergic neurotransmission⁶⁹. Conversely, dopamine agonists (for example, L-dopa and amphetamines) reproduce some of the same symptoms of schizophrenia. Taken together, these results led to the hypothesis that schizophrenia is associated with a hyper-dopaminergic state. However, almost half a century of research has failed to provide solid support for this simple idea. Although neuroleptics treat some of the more dramatic symptoms of schizophrenia, they fail to treat the persistent and equally debilitating symptoms of the disease, including cognitive disorganization and avolition.

The failure of the classic dopamine hypothesis is perhaps not surprising, given our lack of understanding of the role that dopamine has in system-level function. The development of the formal models of dopamine function discussed above, and its interaction with other brain systems, offers hope for a more sophisticated understanding of how dopamine disturbances produce the patterns of clinical psychopathology observed in schizophrenia. For example, along with evidence of dopamine disturbances, it has long been recognized that schizophrenia is associated with disturbances of frontal lobe function. This was originally suggested by comparing disturbances in executive function observed in schizophrenia (for example, distractibility, and cognitive disorganization) with those observed in patients with frontal lobe damage. More recently, neuro-imaging studies have produced more direct evidence of deficits in frontal lobe function, and several investigators have begun to link these deficits with disturbances of dopamine function.

Specifically, schizophrenia may be associated with reduced dopamine activity in frontal cortex coupled with excess dopamine activity in subcortical structures, such as the striatum⁷⁰. Early modelling work showed how a reduction of dopaminergic gain modulation in the PFC can simulate the behavioural deficits observed in patients with schizophrenia⁷¹. The learning and gating functions of dopamine reviewed here suggest ways in which this theory could be elaborated to include specific neuropharmacological findings.

Perspective

Despite our growing knowledge about some of the biological disturbances associated with schizophrenia, as yet there is no biological assay that can be used to diagnose this disease definitively. This reflects the deep limitations in our understanding of the relationship between biological disturbances and their clinical expression as perturbed mental or emotional function. We are entering a time where the formal synthesis of experimental data, both behavioural and physiological, will be needed to address the many open questions surrounding mental illness and behavioural decision-making. □

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Generalization in vision and motor control

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Learning is more than memory. It is not simply the building of a look-up table of labelled images, or a phone-directory-like list of motor acts and the corresponding sequences of muscle activation. Central to learning and intelligence is the ability to predict, that is, to generalize to new situations, beyond the memory of specific examples. The key to generalization, in turn, is the architecture of the system, more than the rules of synaptic plasticity. We propose a specific architecture for generalization for both the motor and the visual systems, and argue for a canonical microcircuit underlying visual and motor learning.

Arguably, the problem of learning represents a gateway to understanding intelligence in brains and machines, to discovering how the human brain works and to making intelligent machines that learn from experience. What distinguishes nontrivial learning from memory is the ability to generalize: that is, to apply what has been learned from limited experience to new situations. Memory bears the same relationship to learning as a dry list of experimental measurements does to a predictive scientific theory. The key question addressed here — from the perspective of the visual and motor systems — is what are the brain mechanisms for such generalization?

Imagine looking for the phone in a new hotel room. Your visual system can easily spot it, even if you have never seen that particular phone or room before. So, learning to recognize is much more than straightforward pixel-by-pixel template matching. Visual recognition is a difficult computational problem, and it is a key problem for neuroscience. The main computational difficulty is that the visual system needs to generalize across huge variations in the appearance of an object; for instance, owing to viewpoint, illumination or occlusions. At the same time, the system needs to maintain specificity; for example, to identify a particular face among many similar ones.

A similar ability to generalize is key to motor learning. Consider practicing how to hit a tennis ball: having learned to play a specific shot, you must then be able to use it under new conditions, adapting to changes in the spin on the incoming ball, the speed and direction of your opponent's shots, the position of your body with respect to the ball, and so on. No two shots can be exactly the same, requiring a generalization ability of our motor program that can involve the modulation of thousands of motor units in new, adaptive ways.

In abstract terms, generalization is the task of synthesizing a function that best represents the relationship between an input, x , and an output, y — an image and its label, say, or a desired equilibrium position of an arm and the set of forces necessary for attaining it — by learning from a set of 'examples', x_i, y_i . In this formulation, the problem of learning is similar to the problem of fitting a multivariate function to a certain number of measurement data. The key point is that the function must generalize. Generalization in this case is equivalent to the ability of estimating correctly the value of the function at points in the input space at which data are not available — that is, of interpolating 'correctly' between the data points. In a similar way, fitting experimental data can, in principle,

uncover the underlying physical law, which can then be used in a predictive way. In this sense, the process of learning distills predictive 'theories' from data; that is, from experience.

The modern mathematics of learning¹ gives a precise definition of generalization and provides general conditions that guarantee it. It also implies that the ability to generalize in the brain depends mostly on the architecture of the networks used in the process of learning, rather than on the specific rules of synaptic plasticity. (The latter are reviewed in this issue by Abbott and Regehr, page 796.)

Here, we highlight a network architecture supporting the ability to generalize in the visual and motor systems. Neurons at various levels of the visual cortex are generally tuned simultaneously to multiple attributes; that is, they respond to a particular pattern of their inputs, and the frequency of the firing follows a 'tuning curve', with a maximum for specific values of each of the attributes (together representing an optimum stimulus for the neuron), such as a particular direction of movement and a specific colour and orientation (Fig. 1 shows tuning for specific object views, each characterized by many parameters; see review in this issue by Tsodyks and Gilbert, page 775). We describe how a linear combination of the activities of such neurons can allow generalization, on the condition that the tuning is not too sharp, and that the weights of such a linear combination are what changes during learning. We then turn to the motor system and show that a linear combination of neural modules — each module involving several motor neurons innervating a coherent subset of muscles which together generate a force field — is mathematically equivalent to the linear combination of tuned neurons described for the visual system. Finally, we propose that the necessarily broad tuning of motor and visual neurons might be based on a canonical microcircuit repeated throughout different areas of cortex.

Generalization mechanisms in the visual system

Older mental models of how vision might work used the simple notion of 'computation through memory'. The classic example is the 'grandmother' theory for vision, in which visual recognition relies on 'grandmother' neurons responding selectively to the precise combination of visual features that are associated with one's grandmother. This theory was not restricted to vision: the same basic idea surfaced for other sensory modalities, for example in motor control, where it is called 'motor tapes'. These ideas were attractive because of their simplicity: they replace complex information processing with the simpler task of accessing a memory.

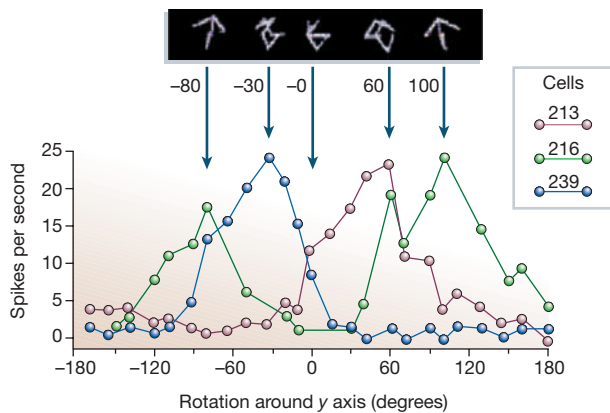


Figure 1 Tuned units in inferotemporal cortex. A monkey was trained to recognize a three-dimensional 'paperclip' from all viewpoints (pictured at top). The graph shows tuning to the multiple parameters characterizing each view summarized in terms of spike rate versus rotation angle of three neurons in anterior inferotemporal cortex that are view-tuned for the specific paperclip. (The unit corresponding to the green tuning curve has two peaks — to a view of the object and its mirror view.) A combination of such view-tuned neurons (Fig. 2) can provide view-invariant, object specific tuning as found in a small fraction of the recorded neurons. Adapted from Logothetis *et al.*¹³.

The basic problem with these models is, of course, generalization: a look-up table cannot deal with new events, such as viewing a face from the side rather than the front, and it cannot learn in the predictive sense described earlier. One of the simplest and most powerful types of algorithm developed within learning theory corresponds to networks that combine the activities of 'units', each broadly tuned to one of the examples (Box 1). Theory (see references in Box 1) shows that a combination of broadly tuned neurons — those that respond to a variety of stimuli, although at sub-maximal firing rates — might generalize well by interpolating among the examples.

In visual cortex, neurons with a bell-shaped tuning are common. Circuits in inferotemporal cortex and prefrontal cortex, which combine activities of neurons in inferotemporal cortex tuned to different objects (and object parts) with weights learned from experience, may underlie several recognition tasks, including identification and categorization. Computer models have shown the plausibility of this scheme for visual recognition and its quantitative consistency with many data from physiology and psychophysics^{2–5}.

Figure 2 sketches one such quantitative model, and summarizes a set of basic facts about cortical mechanisms of recognition established over the last decade by several physiological studies of cortex^{6–8}. Object recognition in cortex is thought to be mediated by the ventral visual pathway running from primary visual cortex, V1, over extrastriate visual areas V2 and V4 to the inferotemporal cortex. Starting from simple cells in V1, with small receptive fields that respond preferably to oriented bars, neurons along the ventral stream show an increase in receptive field size as well as in the complexity of their preferred stimuli. At the top of the ventral stream, in the anterior inferotemporal cortex, neurons respond optimally to complex stimuli such as faces and other objects. The tuning of the neurons in anterior inferotemporal cortex probably depends on visual experience^{9–19}. In addition, some neurons show specificity for a certain object view or lighting condition^{13,18,20–22}. For example, Logothetis *et al.*¹³ trained monkeys to perform an object recognition task with isolated views of novel three-dimensional objects ('paperclips'; Fig. 1). When recording from the animals' inferotemporal cortex, they found that the great majority of neurons selectively tuned to the training objects were view-tuned (see Fig. 1) to one of the training objects. About one tenth of the tuned neurons were view-invariant, consistent with an earlier computational hypothesis²³.

In summary, the accumulated evidence points to a visual recognition system in which: (1) the tuning of inferotemporal cortex cells is obtained through a hierarchy of cortical stages that successively combines responses from neurons tuned to simpler features; and (2) the basic ability to generalize depends on the combination of cells tuned by visual experience. Notice that in the model of Fig. 2, the tuning of the units depends on learning, probably unsupervised (for which several models have been suggested²⁴; see also review in this issue by Abbott and Regehr, page 796), since it depends only on passive experience of the visual inputs. However, the weights of the combination (see Fig. 3) depend on learning the task and require at least some feedback (see Box 2).

Thus, generalization in the brain can emerge from the linear combination of neurons tuned to an optimal stimulus — effectively defined by multiple dimensions^{25,23,26}. This is a powerful extension of the older computation-through-memory models of vision and motor control. The question now is whether the available evidence supports the existence of a similar architecture underlying generalization in domains other than vision.

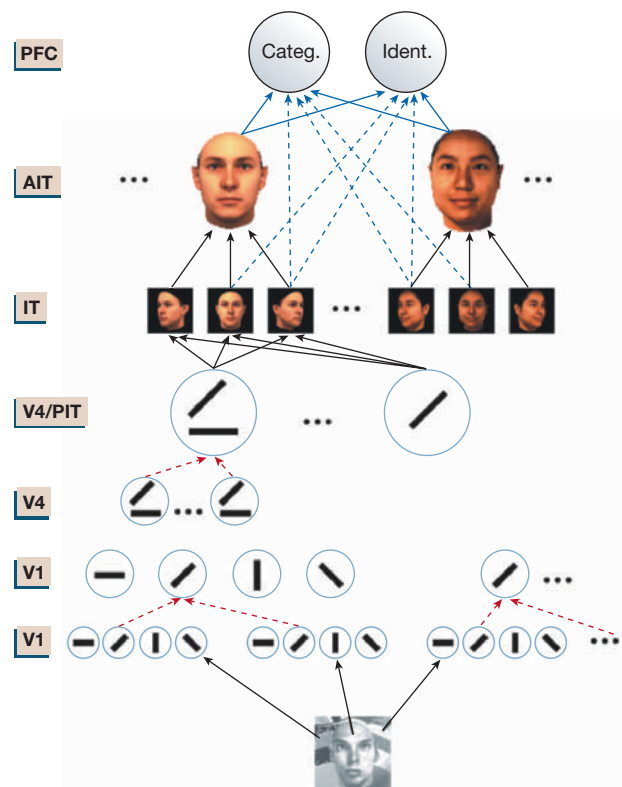


Figure 2 A model of visual learning. The model summarizes in quantitative terms other models and many data about visual recognition in the ventral stream pathway in cortex. The correspondence between the layers in the model and visual areas is an oversimplification. Circles represent neurons and arrows represent connections between them; the dots signify other neurons of the same type. Stages of neurons with bell-shaped tuning (with black arrow inputs), that provide example-based learning and generalization, are interleaved with stages that perform a max-like operation³ (denoted by red dashed arrows), which provides invariance to position and scale. An experimental example of the tuning postulated for the cells in the layer labelled inferotemporal in the model is shown in Fig. 1. The model accounts well for the quantitative data measured in view-tuned inferotemporal cortex cells¹⁰ (J. Pauls, personal communication) and for other experiments²⁵. Superposition of gaussian-like units provides generalization to three-dimensional rotations and together with the soft-max stages some invariance to scale and position. IT, inferotemporal cortex; AIT, anterior IT; PIT, posterior IT; PFC, prefrontal cortex. Adapted from M. Riesenhuber, personal communication.

Box 1

Learning and generalization with tuned, gaussian-like units

Basis functions

The problem of learning from examples can be formulated as a problem of function approximation with the property of generalization (robustness to noise is a special case of the ability to generalize). A classical and simple mathematical approach to solving it is regularization: the function f learned from the data minimizes the error on the training set subject to certain 'smoothness' constraints. An intriguing result is that the solution of the minimization problem above can be expressed as a linear combination of basis functions k centred on the examples $\bar{x}_i$ and depending on the new input vector $\bar{x}$:

$$f(\bar{x}) = \sum_i^n w_i k(\bar{x}, \bar{x}_i),$$

where the $\bar{x}_i$ are the n (vector) examples and w_i are parameters to be determined (for example, learned) from the n example pairs $\bar{x}_i, y_i$, where $\bar{x}_i$ is the 'input' part of each example and y_i is its associated label or 'output'. The basis functions k are fixed functions, such as the gaussian function, of the input. Note that the centres $\bar{x}_i$ (the optimal stimuli for each of the basis functions) are simply learned from 'passive' visual experience without the need of feedback, whereas the weights w_i also depend on the y_i (corresponding to the feedback) and can also be learned by simple learning rules such as the delta rule or the covariance rule⁶⁰. When the basis functions are radial gaussian functions, the network consists of units each tuned to one of the examples with a bell-shaped activation curve. Each 'unit' computes the distance $\bar{x} - \bar{x}_i$ of the input vector $\bar{x}$ from its centre $\bar{x}_i$ (that is, the dissimilarity of the input and the example stored in that unit) and then applies the function k to the dissimilarity value, that is, it computes the function $k(\bar{x} - \bar{x}_i)$. Notice that in the limiting case of $k(\bar{x} - \bar{x}_i)$ being a delta function (for example, a very narrow gaussian function), the network becomes a look-up table, in which a unit gives a non-zero signal only if the input $\bar{x}$ exactly matches its centre $\bar{x}_i$: the network cannot generalize and becomes a simple memory. The equation above can always be rewritten as a feedforward network (Fig. 3a) with one hidden layer containing as many units as examples in the training set. The units of the hidden layer correspond to the basis functions and can be regarded as processors doing a specific operation; the parameters w_i correspond to the weight of the synapses from the units k to the output. The scalar output case above can be generalized to the multi-output case (for example, the approximation of vector

fields), which is the general case and the relevant one for motor control (Fig. 3b). The function $f(\bar{x})$ is thus the superposition of local, tuned receptive fields such as gaussians; it is predictive; and it is also a smooth (the exact definition of 'smooth' depends on k) solution as it minimizes a smoothness constraint such as jerk⁶¹, while being close to the examples. There are alternative ways to implement similar solutions within recurrent networks²⁵. It is well known that regularization networks can be interpreted in bayesian terms^{25,62} but detailed models of how general bayesian networks and graphical models may be implemented in the visual or motor system are lacking so far.

Time

We have described time-independent aspects of visual recognition and motor control, corresponding respectively to recognition of static images and to control of postures (say of an arm). In most of real life, recognition and motor control happen in time: we recognize actions and we control dynamic movements. The equations above, describing the superposition of 'prototypical' images or prototypical force fields, can be extended in time. Possibly the simplest such extension is provided by:

$$\bar{f}(x, t) = Y\bar{b}(x) = \sum_i^n b_i(x) g_i(t) \bar{y}_i$$

where $\bar{f}$ and $\bar{y}_i$ are vector fields and $g_i(t)$ the associated time dependence. This representation seems to be consistent with experimental data in motor control⁵⁴.

A similar description summarizes the model of Giese and Poggio⁵⁵ for the recognition of actions from image sequences. Sequence selectivity results from asymmetric lateral connections between the snapshot neurons in the form pathway (and between the optic flow pattern neurons in the motion pathway). With this circuitry, active snapshot neurons pre-excite neurons that encode temporally subsequent configurations and inhibit neurons that encode other configurations. Significant activity can arise only when the individual snapshot neurons are activated in the 'correct' temporal order. Simulations show that in the model, appropriate lateral connections for the 'correct' sequences can be learned robustly with a simple time-dependent hebbian learning rule from a small number of stimulus repetitions, consistent with psychophysical data⁵⁵.

Generalization mechanisms in the motor system

The architecture for generalization outlined for the visual system (Fig. 3a) leads to a stage of broadly tuned units. For any specific visual recognition task, there are many inputs (such as the photoreceptors) and just one output signal. In the computational architecture of the motor system, however, the flow of information is the opposite, with few inputs (discrete cortical commands from the fronto-parietal cortex) and many outputs (the interneurons and motoneurons in the spinal cord). For such architectures, the combination (with fixed weights set by learning) of neurons tuned by learning to optimal stimuli (with an activity dependent on the similarity between the input and the optimal stimulus) can be formally viewed (see legend of Fig. 3) as a combination (with weights depending on the input signal) of neural circuits or modules, each generating a (fixed) motor 'field' of muscle forces. The non-trivial equivalence may lead to novel experiments. It also suggests that the evidence in the literature about tuned neurons may be fully compatible with the apparently different reports supporting the combination of modules and associated force fields.

In the fronto-parietal cortical areas, arm-related, broadly directionally tuned neurons were first described by Georgopoulos *et al.*²⁷. These neurons are related to arm movements and their tuning means that their frequency of discharge varies in an orderly fashion

with the direction of movement. For each neuron, the discharge was most intense in a preferred direction resulting in a directional bell-shaped tuning curve. In the motor areas of the frontal lobe, neurons with similar preferred direction are interleaved with mini-columns having nearly orthogonal preferred directions²⁸. This recent discovery indicates that the motor cortex is endowed with functional modular structures not unlike those described for the visual cortex^{6,7}, the somato-sensory cortex⁸ and the auditory cortex²⁹. Neuronal activity in the frontal cortical areas, such as the primary motor cortex, the supplementary motor areas and the dorsal premotor areas, change during adaptation and visuo-motor learning^{30,31}, and during exposure to mechanical loads³²⁻³⁴. In addition, during motor learning a significant number of cortical cells change their directional tuning.

While the significance of the information conveyed by the activity of broadly tuned cortical neurons remains hotly debated, here we put forward the hypothesis that the descending cortico-spinal impulses may represent signals (such as the components of the vector $b(\bar{x})$ in Fig. 3) that specify the activation for the modules in the spinal cord of vertebrates. Several kinds of modular spinal systems, consisting of circuits of interneurons, have been described. These range from central pattern generators and unit burst generators³⁵⁻³⁷ to spinal motor primitives generating specific force fields and muscle synergies^{38,39}.

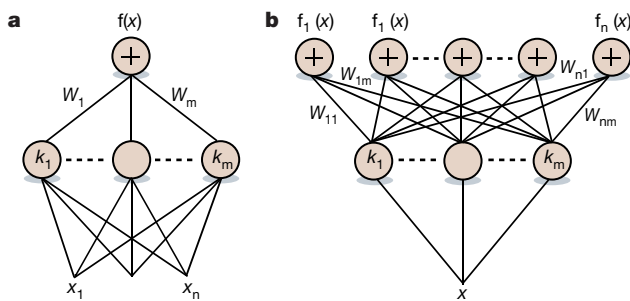


Figure 3 The generalization architectures of the visual and motor systems. **a**, In the case of vision, the single output signal is a combination of tuned unit activities $f(x) = \sum_i w_i k_i(x)$. **b**, In the case of motor control, the output vector can be similarly written as $\vec{f}(x) = \sum_i \vec{w}_i k_i(x)$ where each component of the output field is a combination of tuned unit activities. Here, $\vec{w}_i$ is the vector $w_{i1}, \dots, w_{in}$ of the weights associated with the tuned unit i . The same equation can also be read as a combination of the fields $\vec{w}_i$ with coefficients $k_i(x)$; that is, a combination of fields modulated by the activities of the tuned units. Thus, a combination of tuned units is formally equivalent to a combination of fields. The general description of the networks shown is given, rewriting the last equation, by $\vec{f}(x) = Wk(x)$, where W is a matrix and $k(x)$ is a vector with the tuned units as components. Notice that the factorization in terms of coefficients and basis function is not unique (when only the input and the outputs of the network are observed) since $Wk(x) = Cb(x)$ where L is any matrix satisfying $W = CL$ and $b(x) = Lk(x)$. An additional constraint (such as specifying which parameters of the network change with learning) is needed to fix L . The arbitrariness in the decomposition might explain apparent differences in the interpretations of some experiments. For instance, Thoroughman and Shadmehr⁵⁰ conclude from behavioural data that the basis functions are gaussian and tuned to desired velocities, whereas cortical cells would presumably show a linear tuning as a function of velocity²⁷.

Box 2

Supervised and semi-supervised online learning

The distinction between supervised and unsupervised learning in biology can be tricky: there is a whole spectrum between the two. The learning process by which the neurons in the model of Box 1 get tuned more and more specifically to a particular value of a given attribute is unsupervised: it relies only on the inputs, and it does not require feedback about the correctness or incorrectness of the output. Several mechanisms have been suggested for such unsupervised learning of tuned units²⁴ (see also review in this issue by Abbott and Regehr, page 796). By contrast, the coefficients of the linear combination of the unit responses (labelled w_i in Box 1 and Fig. 3a), similar to the synaptic weights in neural networks, depend on the task and require at least some feedback about the output — for example, whether the ‘male face’ label was the correct answer or not. By definition, therefore, the modification of the weights during training is a supervised form of learning. The visual and motor tasks described in this paper are mostly supervised in the laboratory: for each example x (input), there is a label y (correct output); in experiment, monkeys receive feedback in every trial during training. Semi-supervised ‘online’ learning, however, in which feedback is provided for only some of the examples, is a better description of real-life visual and motor learning (see review in this issue by Tsodyks and Gilbert, page 775). Note that the full unsupervised learning problem (technically called density estimation) can be solved using supervised learning algorithms⁵⁶. Furthermore, it turns out⁵⁸ that extending the regularization networks described in Box 1 to the unsupervised case is natural and does not change the basic architecture⁵². Biological learning is usually sequential and can therefore be characterized as online learning, in which the examples are provided one at a time. For online learning, biologically plausible versions of stochastic gradient descent can be used²⁵ (see review in this issue by Abbott and Regehr, page 796).

Because limbs are typically controlled by multiple sets of muscles (and an even larger number of muscle motor units), a major challenge in motor control has been to explain how the cortical cells modulate signals out of such large search space so that functional movements are generated. Previous work in vertebrates and invertebrates supports our hypothesis above, suggesting that specific motor behaviours are constructed through flexible combinations of a small number of modules, each generating a force field (in vertebrates a module is composed of a population of interneurons^{40,41}, but in invertebrates a single interneuron may function as a module⁴⁰). According to this view, a module may reduce the number of degrees of freedom by controlling groups of muscles — and thus the associated field of forces — thereby functioning as a computational unit for use with different modulations in multiple motor behaviours^{40,42,43}. Perhaps the most interesting aspect of the work was the discovery that the force fields induced by the focal activation of the cord follow a principle of linear combination^{39,44} (see legend of Fig. 3 and Fig. 4), although this does not seem to hold for cats⁴⁵). Specifically, Mussa-Ivaldi *et al.*³⁹ stimulated simultaneously two distinct sites in the frog’s spinal cord and recorded the resultant forces at the ankle. They observed vector summation of the forces generated by each site separately: when the pattern of forces recorded at the ankle following co-stimulation were compared with those computed by summation of the two individual fields, they found that ‘co-stimulation fields’ and ‘summation fields’ were equivalent in more than 87% of cases. This is also true in the rat⁴⁶. Moreover, the force-field summation underlies the control of limb trajectories in the frog⁴⁷.

Thus the hypothesis for explaining movement and posture is based on combinations of a few basic fields. The force fields (corresponding to the columns of the matrix C in the legend of Fig. 3) stored as synaptic weights in the spinal cord may be viewed as representing motor field primitives from which, through linear superimposition, a vast number of movements can be fashioned by impulses conveyed by supraspinal and reflex pathways. Computational analysis⁴⁸ verifies that this proposed mechanism is capable of learning and controlling a wide repertoire of motor behaviours.

Additional support to this view was provided by behavioural studies of reaching movements showing that when new forces are encountered, primates learn new dynamics to implement the desired trajectory⁴⁹. Thoroughman and Shadmehr⁵⁰ were able to conclude from the pattern of generalization that the desired velocity of the reaching hand is mapped into a force required to move the hand at this velocity by combining tuned units with a gaussian shape. Their model can also be described in an equivalent, dual way as a combination of force fields (Fig. 3; Box 1).

In conclusion, there is independent evidence, in separate studies, for tuned neurons in motor cortex, and for a combination of a limited number of basic modules, each generating a force field and each modulated by supraspinal signals, in agreement with the caricature of Fig. 3b.

A canonical local circuit for tuning and generalization?

Thus, it seems that the combination of tuned receptive fields is the basic strategy used by both the visual and motor systems to learn and generalize. The similarity of the strategies in the visual and motor cortex, suggests that they might occur in other systems where learning is a component. The circuits that underlie the bell-shaped tuning curves are not known. Many cortical neurons seem to be tuned to a specific pattern of inputs, meaning that the maximum response of the cell occurs when the set of inputs takes specific activation values (which in general are not the set of maximum values of each input). It is a puzzle how this multidimensional tuning could be obtained parsimoniously by plausible neural circuits. One possibility is that tuning of a neuron to a specific set of activities of its many inputs (an infratemporal cortex neuron is likely to receive inputs from many cells, for instance from V4) is achieved by normalizing the inputs, which means dividing each one by the sum of the strengths of all of them. In fact, gaussian-like, multidimensional tuning — as found in many neurons in cortex —

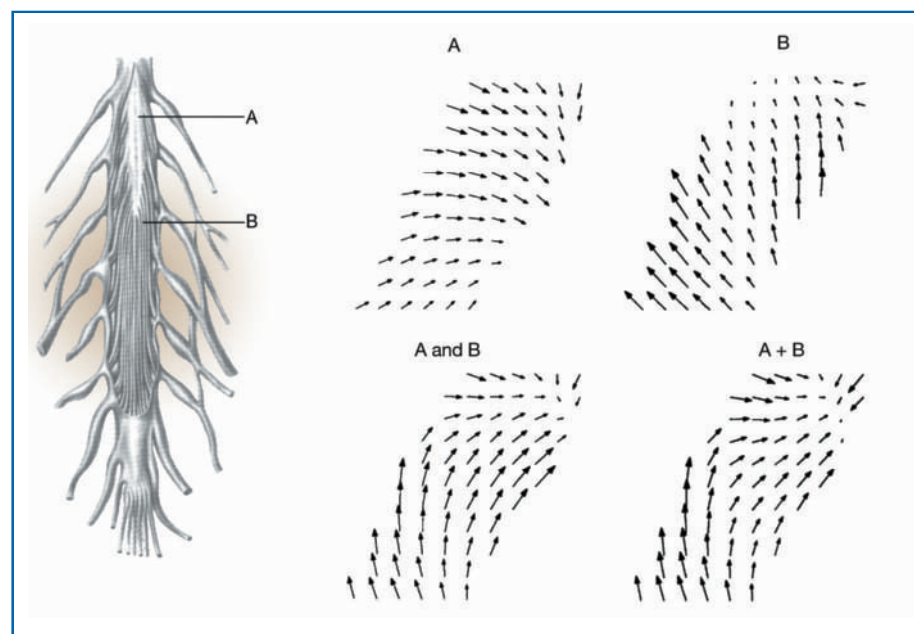


Figure 4 Spinal force fields combine linearly. Force fields A and B were obtained in response to stimulations delivered to two different spinal sites. The 'A and B' field was obtained by stimulating the same two sites simultaneously. It matches closely (correlation coefficient larger than 0.9) the force field A+B, which was calculated by pair-wise adding of the vector fields in A and in B. This highly linear behaviour applies to more than 87% of dual stimulation experiments. Adapted from Mussa-Ivaldi *et al.*³⁹.

can be generated by normalization of the input vector, followed by a simple threshold-like sigmoidal nonlinearity (Box 3).

Various neural circuits have been proposed to implement the key normalization stage, although the motivation behind the suggestions was to account for gain control and not tuning properties^{51,52} (see review in this issue by Destexhe and Marder, page 789). Here, we propose that another role for normalizing local circuits in the brain is to provide (multidimensional) gaussian-shaped tuning, as a key step towards generalization. In fact, this might be the fundamental reason for the widespread presence of gain control circuits in cortex, where tuning to optimal stimuli is a common property. The normalization circuits might, for instance, use recurrent inhibition of the shunting type (Box 3), for which there is abundant evidence in cortex⁵³, although this is only one of several possibilities. Interestingly, the same basic circuit could implement the soft-max operation proposed for some of the processing stages in the visual system (Fig. 2). In any case, our new hypothesis is that gain control microcircuits underlie the tuning of cells to optimal stimuli in both the visual and motor systems.

Further questions in neuroscience and learning theory

Computational models versus experiments

Throughout this review, we used theoretical models as a tool to summarize experimental data provided by different approaches. The problems of visual recognition and motor control are computationally difficult and the experimental data from different sources are growing rapidly. We believe that quantitative models will increasingly replace the traditional qualitative mental models of the visual and motor physiologist and will become ever more important tools for interpreting data, and for planning and analysing experiments.

Time in vision and motor control

Our discussion of the visual system concentrated on the special case of recognition of a static image. In reality, we can recognize images that move and even sequences of movements. In the motor system, time has an even more obvious role: most of our motor commands deal with time-dependent motions and not simply with static postures. In vision, time can be introduced in a direct way assuming

that visual neurons react to 'snapshots' of a motion and are selective for sequences of snapshots. In motor control, the equivalent assumption is that the motor primitives are time dependent. Box 1 suggests a strong analogy between vision and motor control in the time-dependent case: the basic strategy is to combine locally tuned units with time-dependent properties^{54,55}.

Hierarchical cortex architectures

It seems that modern learning theory does not offer any general argument in favour of hierarchical learning machines. This is a puzzle because the organization of cortex — as we argued for the visual and motor cortex — seems to be hierarchical.

Why hierarchies? There could be reasons of efficiency — computational speed and use of computational resources. For instance, the lowest levels of the hierarchy in visual cortex might represent a dictionary of features that can be shared across multiple classification tasks⁵⁶. Hierarchical systems usually break down a task into a series of simple computations at each level. The same argument could apply to motor cortex. There might also be a more fundamental issue. Classical learning theory shows that the difficulty of a learning task depends on the complexity of the

required learning architecture. This complexity determines in turn how many training examples are needed to achieve a given level of generalization. Thus, the complexity of the learning architecture sets the sample complexity for learning. If a task such as visual recognition can be decomposed into low-complexity learning tasks, for each layer of a hierarchical learning machine, then each layer might require only a small number of training examples. Of course, not all tasks have a hierarchical representation. Roughly speaking, the issue is about compositionality (S. Geman, personal communication): neuroscience suggests that what humans can learn — in vision and motor control — can be represented by hierarchies that are locally simple. Thus, our ability to learn from just a few examples, and its limitations, might be related to the hierarchical architecture of cortex.

Learning from very few examples

How then do the learning machines described in modern learning theory compare with brains? There are of course many aspects of biological learning that are not captured by the theory and several difficulties in making any comparison. One of the most obvious differences is the ability of people and animals to learn from very few examples. This is one of the challenges for the learning architectures we propose (although the networks described here, in particular the network of Fig. 2, can learn certain recognition tasks from less than ten labelled examples; M. Riesenhuber, personal communication). Of course, evolution has probably done a part of the learning and encoded it in the DNA. For instance, there is some evidence for basic face categorization ability to be present in human infants at birth, and for face-tuned neurons to be present in inferotemporal cortex of infant monkeys⁵⁷.

In any case, neuroscience suggests that an important area for future work on the theory and on the algorithms, is the problem of learning from partially labelled examples (and the related area of active learning): biological organisms usually have much visual and motor experience but mostly without direct or indirect feedback (providing the labels). Interesting theoretical work has begun on this; for example, showing that regularization networks (similar to the combination of tuned cells) could update their coefficients from a

Box 3

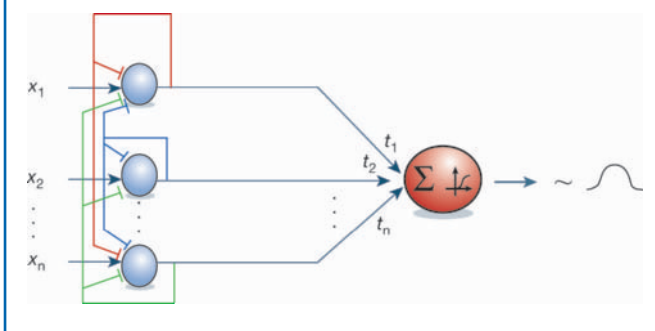
A neural circuit for gaussian-like tuning

For normalized t and x vectors (in the euclidean norm), the sigmoid of a scalar product can approximate a gaussian-like radial function⁶³. Among the various neurally plausible circuits that have been proposed to approximate a normalization stage⁵¹, we describe here a specific circuit, using lateral shunting inhibition^{64–66}, mainly to provide a possible example. There are certainly different possibilities for the nervous system to implement local normalization modules; for instance, using more complex synaptic properties (see review in this issue by Abbott and Regehr, page 796. The simplest equation — here in a time-independent form — describing a feedforward network of lateral shunting inhibition has the following form in a network of n cells:

$$y_i = \frac{h(x_i)}{c + \sum_k k(x_k)} \quad i = 1, \dots, n.$$

where h and k represent the transduction between nonlinear presynaptic and postsynaptic voltage at the output of cell i and at the output of the interneurons mediating lateral inhibition, respectively. If $h(x) = x$ and $k(x) \equiv \sqrt{x^2}$, the circuit performs a normalization operation; if $h(x) \equiv x^{q+1}$ and $k(x) \equiv x^q$ with q sufficiently large ($q \geq 2$), then the circuit performs a max operation, for example, $y_i \equiv x_i$ if $x_i = \max_j x_j$, otherwise $y_i \equiv 0$ (see ref. 67). The figure shows the circuit with inhibition of the shunting type (the arrows indicate depolarizing synapses, whereas the symbol $\dashv$ indicates shunting inhibition onto interneurons (blue). Depending on the parameters, the activity of the tuned output cell (red) — after summation of the inputs with $x_1, x_2, \dots, x_n$ weighted with synaptic weights $t_1, t_2, \dots, t_n$ and then transformation through a sigmoidal, threshold-like nonlinearity, such as provided by the spike mechanism — can approximate a gaussian-like, bell-shaped function of the inputs, that is $e^{-(x_i - t_i)^2 - (x_i - t_i)^2 \dots - (x_i - t_i)^2}$, since the input vector is normalized by the recurrent inhibitory circuit.

Note that the neuron responds maximally to the 'optimal' pattern of inputs with values $t_1, t_2, \dots, t_n$. Note also that the same basic circuit of lateral inhibition with somewhat different synaptic parameters could underlie gaussian-like tuning (by means of normalization) and the softmax operation⁵⁴ — which are the two key operations required at various stages in the model of object recognition shown in Fig. 2.



partially labelled set of examples⁵⁸. Other approaches, such as bayesian and graphical models, might be able to deal more generally with the problem of unsupervised learning (for example, ref. 25).

The mind as a theory of the world

In modern mathematical theory, the property of generalization is the key property of learning. Learning, as opposed to memory, synthesizes functions that are predictive of the world. Thus, learning synthesizes modules — such as vision and motor control — that are effectively theories of the physical world, in the sense of being predictive of specific aspects of it. Learning is done within these architectures by the plasticity of synapses, and learning is what makes the brain a theory of the world.

The quest for generalization mechanisms

There is considerable evidence in the visual and motor system for the learning architectures we propose — a combination of tuned units. But whether our hypothesis is a satisfactory, first-order description of the first few hundred milliseconds of visual perception and motor control or whether more complex, recurrent network models will be needed remains unclear. It will be interesting to look at other systems, such as the auditory, somatosensory and olfactory systems, from a similar point of view. There is little evidence at this point for or against our proposal of a canonical microcircuit underlying tuning in many neurons throughout the brain^{52,59}; there is even less evidence for the specific circuit we suggest. In fact, other plausible neural and synaptic circuits could work as well. Finally, it is unclear whether similar, simple learning architectures could have any role in typical human brain functions such as learning language. □

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Neural networks and perceptual learning

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Sensory perception is a learned trait. The brain strategies we use to perceive the world are constantly modified by experience. With practice, we subconsciously become better at identifying familiar objects or distinguishing fine details in our environment. Current theoretical models simulate some properties of perceptual learning, but neglect the underlying cortical circuits. Future neural network models must incorporate the top-down alteration of cortical function by expectation or perceptual tasks. These newly found dynamic processes are challenging earlier views of static and feedforward processing of sensory information.

Perceptual learning is the improvement in performance on a variety of simple sensory tasks, following practice. In visual perception, such tasks, often called discrimination tasks, involve identifying small differences in simple visual attributes, such as position (Fig. 1), orientation, texture or shape. In general, perceptual learning ranges from the discrimination of simple attributes to more complex sensory patterns. At one end of the spectrum, it can involve discrimination of visual orientation or depth, auditory pitch, or changes in tactile vibration frequency. At the other end, it can involve detection of geometric shapes or alphanumeric characters^{1,2}.

In perceptual learning, the improvement develops progressively over many trials, as opposed to other, more explicit types of learning which may require only a single exposure to a stimulus. Perceptual learning is implicit, subjects are not consciously aware of it and it progresses even in the absence of a reward for correct responses. Perceptual learning unfolds automatically on repeated exposures to the sensory stimulus, and from integrated efforts at discrimination over a long time. Perceptual learning has important advantages as a brain process amenable to scientific study. First, the behaviour can be quantified relatively accurately under well-defined experimental conditions. Second, there are good reasons to believe that perceptual learning is mediated by neuronal processes that occur at the level of the primary sensory cortex. These areas are the first to receive information from the sensory organs and their circuitry is the best understood of that in the cerebral cortex. Perceptual learning can therefore be quantitatively assessed using three approaches: psychophysical measurement of behaviour, physiological recording of living cortical neurons and computer modelling of well-defined neuronal networks.

Any model of perceptual learning must include at least two components. First, it has to describe the way the sensory world is represented by neuronal activity in the sensory areas of the brain. Second, it has to describe the changes that occur in the sensory pathways when perceptual learning occurs. The current consensus stipulates that every sensory attribute is represented by population activities in the early sensory areas that are dedicated to this attribute. For example, the orientation of visual stimuli is represented by a population of orientation-sensitive neurons in the primary visual areas; the pitch of tonic sounds is represented by a population of frequency-selective neurons in the primary auditory cortex, and so on. The output of such population activity is then

interpreted by higher-order cortical areas, which make perceptual decisions. Much less is known about the nature and location of the changes that underlie the improved performance in a sensory task, although evidence is accumulating that the same early stages in sensory processing that initially represent an attribute also mediate the changes involved in improving the discrimination of that attribute. A daunting challenge posed by this picture is to understand how primary circuits can undergo repeated changes that result from learning, but simultaneously be able to operate in tasks that have already been learned.

Here, we review a few representative models of neural networks and assess their performance in terms of perceptual learning. 'Feedforward networks', although based on a very limited number of input units, provide specific read-outs that improve very specifically and quickly during training. The main drawback of feedforward networks, however, is that they rely on a feedback teaching signal, which does not fit with known brain neuroanatomy. By contrast, 'recurrent networks' rely on more realistic horizontal connections, which allows them to learn without the need for any reinforcement signals. Recurrent network models, however, perform relatively poorly on specific perceptual tasks. Models that combine both feedforward and recurrent architectures address some of these problems, but current models are a long way from matching biological circuits.

In the second section of this review, we discuss in more detail the defining characteristics of perceptual learning, as it occurs in real brains. For each property, we consider the challenges it presents for future modellers. In particular, models must accommodate the effect of top-down influences of attention, expectation and perceptual task on the operation of intrinsic cortical circuits. It is becoming increasingly clear that both the encoding and the retrieval of learned information is dependent on feedback interactions between higher- and lower-order cortical areas in sensory cortex. Models should allow for learning in the absence as well as in the presence of reward feedback. They need to account for the high degree of specificity that perceptual learning is known to have. They also need to allow the same circuits to undergo the changes required to encode learned information without this disrupting their existing role in the analysis of the sensory environment. Finally, the rules and characteristics of cellular plasticity have to be integrated at the synaptic, neuronal and network levels (see review in this issue by Abbott and Regehr, page 796) to fully account for the mechanisms underlying perceptual learning.

Neural network models of perceptual learning

Models of perceptual learning can be broadly divided into two classes: feedforward versus feedback or recurrent network models. These differ in: (1) network architecture; and (2) the location of the functional changes (output versus input levels, respectively³). In feedforward networks (for example, Fig. 2) neurons are located in distinct consecutive layers such that information flows unidirectionally from one layer to another, and learning is implemented by appropriate changes in the relative strengths of feedforward connections. The trigger for changing the connections is usually a discrepancy between the activity at the upper layer (output) and a 'desired' output, which has to be provided to the network during learning ('supervised learning'). In the feedback networks (for example, Fig. 3) information can propagate in loops within a layer or be transferred from higher to lower layers. Such networks allow learning without the

need for a reward or any 'teaching signal' ('unsupervised learning'). A combination of both architectures has been introduced in some models⁴. Indeed, in complete brains most of the sensory areas have the role of read-out for the previous levels and input representation for the subsequent levels of processing.

Feedforward networks

The best known model of the feedforward type is that conceived by Poggio *et al.*⁵ on visual hyperacuity. Poggio *et al.*⁵ proposed a three-layers feedforward network (Fig. 2b), the input layer of which consists of a small number of gaussian filters (receptive fields) that transform any input pattern into a vector of activity levels by convolving the input with the corresponding receptive field profiles. The next layer of the network is a set of radial basis functions, each computing the weighted distance between the input vector and a certain template vector that is unique for each function. Finally, the output of the module is computed as a linear combination of the radial basis functions. In models of vernier discrimination, where subjects determine the direction of offset of one line relative to a nearly collinear reference line, the output value of the model determines the perceptual decision, with positive and negative values of output unit being interpreted as the direction to which the target is shifted relative to the reference.

When the model is trained on a set of example inputs with known outputs, the input receptive fields do not change, but the number of radial basis functions and internal parameters of the network are updated. Surprisingly, with only a very limited number of input receptive fields (eight), the model reproduces some salient properties of perceptual learning with high fidelity. The model's hyperacuity level of performance is similar to the experimentally measured one. This increases with the length of the two segments of the vernier stimulus and is specific to its orientation, all in accordance with psychophysical observations.

The main appeal of Poggio *et al.*'s⁵ model is that it raises the possibility that when a certain perceptual task is practiced, the brain quickly synthesizes a specialized neural module that reads out the responses in the primary sensory areas of the brain in a way that is optimal for this particular task. Because the responses of sensory neurons are not affected by learning and the synthesized module is not involved in any other tasks, the obtained improvement in the performance is highly specific to the task that was practiced. The model also very successfully replicates observed performance under various stimulus manipulations. However, this class of model has some drawbacks. First, because different elements of the input pattern do not interact with each other directly, the ability of the read-out module to discriminate between different inputs does not strongly depend on the spatial shape of the inputs. For example, the model learns to estimate the horizontal displacement of a single vertical bar relative to an arbitrary reference position, with an absolute precision that is similar to that obtained for a corresponding vernier stimulus (Sahar-Pikielny *et al.*, unpublished data). The fact that spatial features of vernier stimuli seem to be crucial for hyperacuity indicates the involvement of lateral interactions between the receptive fields that respond to different components of the stimulus. Second, most of the learning algorithms in feedforward networks, including the one used by Poggio *et al.*⁵, require a teaching signal. Yet, perceptual learning does not require a feedback to proceed, although without it learning proceeds at a slower pace⁶. Third, for a vernier task, human subjects show hyperacuity on the very first trials, which may not leave time for the synthesis of a specialized module.

Recurrent networks

Several recent observations indicating that perceptual learning results in specific changes in the corresponding primary sensory areas, both on the functional level and on the level of single neuron response properties, provide strong support for models based on recurrent networks. An example of this type of model is that proposed by Adini and colleagues^{7,8} which describes perceptual learning in the

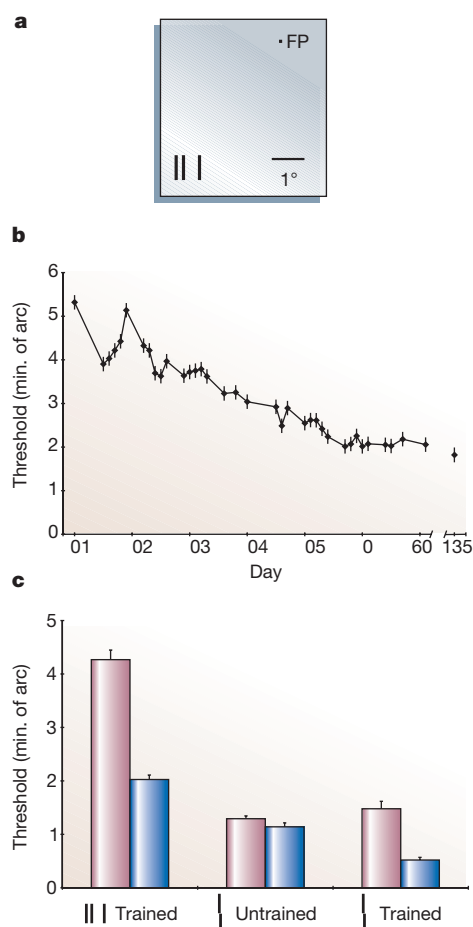


Figure 1 Example of perceptual learning involving a three-line bisection task. **a**, The subject has to determine whether the central line of three parallel lines is nearer the line on the left or on the right. FP indicates the position of the fixation point. Horizontal line shows one degree of viewing angle. **b**, Practicing this task over thousands of trials for many weeks produces a threefold improvement in the 'threshold' — the amount of offset from the central position required to correctly judge the direction of offset. Minute of arc is a 60th of a degree of a viewing angle. The task is practiced in one visual field position, and the improvement is relatively specific to the trained location and orientation, suggesting the involvement of early stages in the visual cortical pathway, where receptive fields are smallest and orientation tuning sharpest. **c**, Importantly, the training is specific for context or the spatial configuration of the stimulus; improvement in the discrimination of the position of a line relative to two parallel lines (three-line bisection) does not transfer to discriminating the position of the same line relative to a collinear line (vernier discrimination). Adapted from ref. 21.

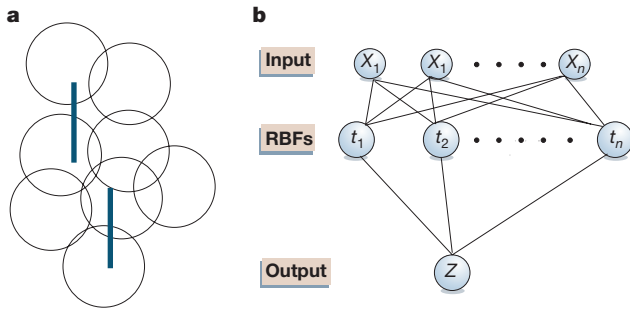


Figure 2 Three-layer feedforward network model of a vernier hyperacuity task. Subjects are required to detect the spatial displacement between the two line segments shown in **a**, superimposed on the receptive fields of the input gaussian filters. The network is shown in **b**. Gaussian filters transform any input pattern into a vector of activity levels by convolving the input with the corresponding receptive field profiles: $x_i = G_{\sigma}(r - r_i) / I(r)$, where $I(r)$ is an amplitude of a visual input at retinal location r and r_i is the centre of the corresponding receptive field. The next layer of the network is a set of radial basis functions (RBFs), each computing the weighted distance between the input vector and a certain template vector that is unique for each function: $y_a = G(|x - t_a|_w)$, where $|x - t_a|_w = (x - t_a)^T W (x - t_a)$ is the weighted distance between the input vector and the template for the function. W denotes the vector of corresponding weights. Finally, the output of the module is computed as a linear combination of the radial basis functions with coefficients: $z = \sum_a c_a y_a$. This output value determines the perceptual decision, for example, positive and negative values of z are interpreted as opposite senses of the vernier displacement. Adapted from ref. 5.

case of contrast discrimination. Adini *et al.*⁷ assume that perceptual learning is mediated by an increase in contrast sensitivity. This, in turn, results from stimulus-evoked modifications to recurrent connections in the local network in the primary visual cortex.

The model assumes that contrast discrimination is mediated by a local cortical column consisting of two interconnected subpopulations of excitatory and inhibitory neurons⁹ (Fig. 4). The activity of the excitatory (E) and the inhibitory (I) subpopulations is determined by the external feedforward inputs (e and i , respectively), which increase with the stimulus contrast (C), and by the strength of recurrent interactions in the local network (J s). Moreover, sensory input from the eye is divided by a fixed proportion between the two populations ($i \equiv ke$; where the constant k does not depend on the contrast).

The contrast discrimination threshold is controlled by the steepness of the relationship between the activity (E) and the contrast; that is, by contrast sensitivity. The synaptic learning rule chosen guarantees the convergence of the synaptic strengths to an equilibrium level after repeated presentations of the stimulus. This equilibrium depends on the way the inputs are divided between the populations (that is, on the value of the constant k), but not on the contrast of the stimulus. So, after the stimulus is seen many times, the network adapts to its configuration and terminates the synaptic modifications. However, surrounding the target stimulus with flankers may rekindle the modifications if the additional input to the target, mediated by intracortical connections, is divided differently between the two populations (that is, if it has a different value for k). To explain the psychophysical results Adini *et al.*⁷ assumed that in the presence of flankers the intracortical input is biased in favour of the inhibitory component more than the feedforward input. If this is the case, practicing the contrast discrimination task in the presence of flankers leaves the local network with higher contrast sensitivity than before practice.

An attractive feature of Adini *et al.*'s⁷ model is that it does not require a feedback teaching signal because synaptic modifications are activity-dependent in a hebbian sense. (A hebbian rule of synaptic modification refers to the idea that synapses between neurons that

are simultaneously active become stronger.) However, the model cannot easily account for the task-specificity of perceptual learning.

The problem of having perceptual learning affect general processing mechanisms is shared by any model of perceptual learning based on activity-dependent modifications in the lateral connections in the primary sensory areas¹⁰. A further example is Teich and Qian's¹¹ model of learning orientation discrimination. The goal of this model was to propose a mechanism for experimentally observed changes in orientation tuning of monkey V1 cells that are specific to the trained orientation¹². (The model is based on the well-studied recurrent model of orientation selectivity proposed in refs 13–15.) Teich and Qian¹¹ demonstrate that observed changes in orientation tuning are reproduced in the model if intracortical excitatory connections to cells at and near the trained orientation weaken slightly as a result of learning. In particular, the tuning curves of cells (neurons' responses as a function of a change in the stimulus) whose preferred orientation is near the trained one becomes sharper, in contrast to the broadened tuning curves of cells whose preferred orientation is farther away from the trained one. Similar manipulations, but ones that involve weakening of both excitatory and inhibitory connections around the trained orientation, lead to effects that are observed during the so-called tilt illusion and adaptation experiments^{16,17}, including iso-orientation inhibition and changes in orientation tuning bandwidth. These two modifications in tuning lead to opposite effects on the orientation discrimination at the trained or adapted orientation (improvement for learning and deterioration for adaptation). An important issue for future studies on synaptic plasticity and its relationship to perceptual learning is the incorporation of mechanisms that guarantee synaptic modifications that lead to improvement in performance during training. Indeed, we know of only one report of practice-induced deterioration in performance¹⁸, which indicates that in general, practice leads to an improvement in performance.

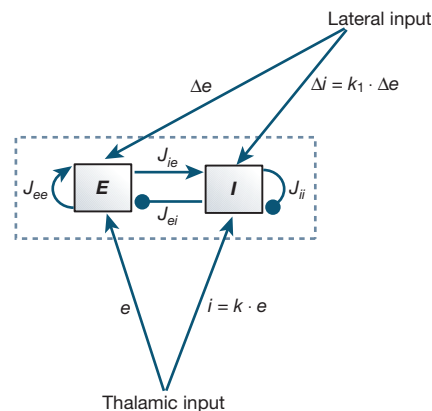


Figure 3 A schematic representation of a cortical column, consisting of two interconnected excitatory (E) and inhibitory (I) subpopulations, with modifiable intrinsic connections — used here to model contrast discrimination. Both E and I populations receive external feedforward inputs from the thalamus (e , i respectively) and from within the cortex (Δe , Δi) when surrounding stimuli are present. Thalamic input increases with contrast but the ratio between e and i remains fixed ($k = \text{constant}$). The resulting network activity also increases with contrast. Assuming the threshold-linear gain functions for both subpopulations, their activity is given by:

$$E = e \frac{1 + J_{ii} - kJ_{ei}}{J_{ei}J_{ie} - (J_{ee} - 1)(J_i + 1)}; I = e \frac{J_{ie} - k(J_{ee} - 1)}{J_{ei}J_{ie} - (J_{ee} - 1)(J_i + 1)}$$

(see ref. 8 for more details). J refers to a strength of corresponding connections. The form of the contrast sensitivity in the network is therefore determined by the feedforward input e , with network interactions providing additional scaling factors. The equilibrium strength of intrinsic connections reached after repeated presentation of the central stimulus alone depends on k but not on the stimulus contrast.

Combined models

Zhaoping *et al.*⁴ proposed a model that combines both recurrent and feedforward learning. This model aims to explain the ability of observers to solve a bisection task with very high precision. Zhaoping and colleagues⁴ demonstrate that a linear feedforward mechanism can account for the observed performance provided that the retinal position of the stimulus array is fixed. This condition, however, is too restrictive. First, experimentally, the effects of learning persist when the stimulus is presented up to several degrees away from the trained position. Second, fixation errors, eye tremor and microsaccades are inevitable over the course of the experiment. As shown by Zhaoping *et al.*⁴, these uncertainties in the exact position of the stimulus lead to a very poor performance of the purely feedforward read-out mechanism. Zhaoping and colleagues propose that this problem can be rectified if the stimulus undergoes recurrent pre-processing based on the horizontal connections in the primary visual cortex. The pattern of this connection has to be chosen in a way that is highly specific to the particulars of the task, such as the range of stimulus array positions and the distance between the stimulus components. If this is done, the bumps of activity that are evoked by each bar of the stimulus are shifted laterally in such a way as to facilitate the consequent perceptual decision mediated by the feedforward mechanism that reads out the activity in the primary visual cortex.

Although recurrent networks provide a more realistic setting as a substrate for perceptual learning, training them to produce an optimal performance on a task is in general an unsolved problem. An interesting approach has recently been proposed by Seung¹⁹, which applies the well-known reinforcement learning algorithm²⁰ to biologically realistic neural networks. The learning algorithm derived by Seung¹⁹ uses the stochastic nature of the synaptic transmission,

which is mediated by probabilistic release of neurotransmitter. According to this algorithm, connections that show a consistent correlation between the neurotransmitter release and good overall performance of the network on the task are 'rewarded' by having their release probabilities increased. Importantly, this idea can be applied to networks with arbitrary architectures, having both feedforward and recurrent elements. However, reaching an optimal performance is crucially dependent on the global evaluation signal (reward) that is available to the synaptic connections in the network.

Real brains' challenges to models

Perceptual learning is highly specific

If a subject is trained on a discrimination task at one location in space, the improvement in performance is relatively specific for that location and does not transfer to other locations in the sensory map. For example, training a subject on a three-line bisection task leads to improvement at the trained location, but the degree of transfer drops off as the visual stimulus is shifted to locations up to 8° away, and there is no transfer when the stimulus is shifted to the opposite hemifield. The training is also specific to the orientation of the trained stimulus. This suggests the involvement of early stages in cortical processing (such as primary visual cortex, V1), where the receptive fields are smallest, the visuotopic maps most highly organized, and the orientation selectivity sharpest²¹. However it is interesting to note that the degree of transfer observed is larger than the receptive fields in V1. This amount of spread of learned information should inform the implementation of computational models of learning.

Perceptual learning is also specific for context and the configuration of the stimulus learned in training. For example, training on a three-line bisection task (Fig. 1) does not transfer to a

vernier discrimination task. In both tasks the target of the discrimination has the same visual field position and orientation, and the trained attribute (position) is also the same. But in one task the context is two side-by-side flanking parallel lines and in the other it is two lines that are collinear with the target. Contextual specificity has been seen in other forms of perceptual learning, such as depth discrimination²². It is worth noting, however, that nonspecific effects of perceptual learning on the basic representations within an adult visual system have recently been reported for amblyopic patients²³. But the more general rule is that learning on one task only shows transfer to another task to the degree that both tasks have elements in common. Further work is needed to determine — when training in discriminating multiple stimuli — which components of these stimuli are employed for making the discrimination. Models will assume greater importance in guiding these studies by showing which features are most useful for recognition systems to generalize to novel stimuli²⁴.

The observed task specificity of perceptual learning poses a serious challenge to models based on changes in the wiring of neural circuits in the primary sensory areas. This is because task specificity should lead to some general effects on sensory processing in the particular domain that is affected by training. An exciting possibility that could explain the relative absence of cross-talk could be a task-dependence of the lateral interactions in the sensory areas. Indeed, after a monkey was trained on a three-line bisection task, the modulation of the cell's response to a line segment within the receptive field by a second parallel line, placed outside the receptive field, differed depending on whether the monkey was tested on the trained task or on an unrelated fixation or vernier discrimination task^{25,26}.

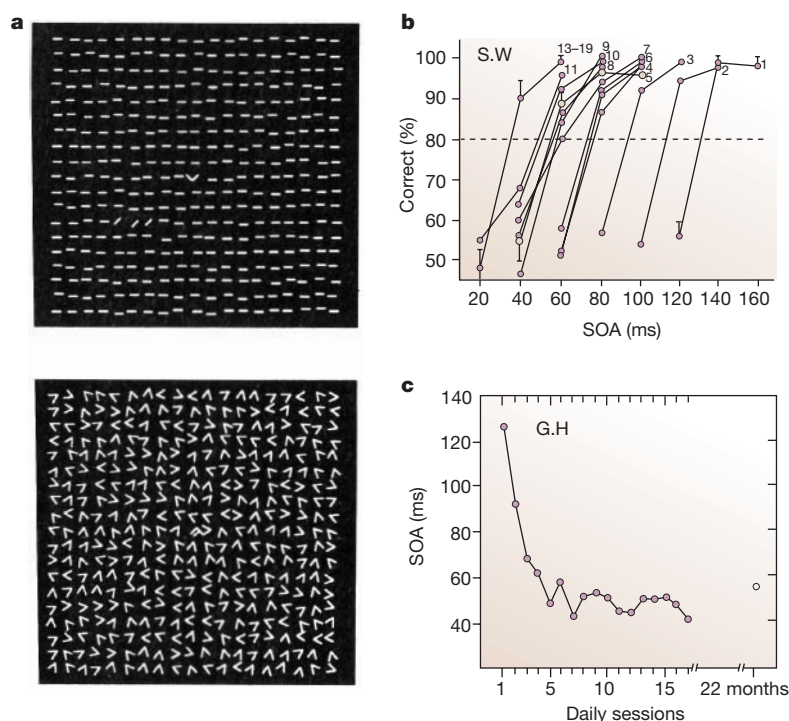


Figure 4 Learning on a texture discrimination task. **a**, The subject views a pattern of lines and has to detect the orientation of a series of lines that are presented at a different orientation from the background lines (top). The stimulus array is followed by a 'mask' (bottom) after different delays (SOA or stimulus onset asynchrony). **b**, Improvement is measured as a shortening of the delay time to mask presentation that still allows 80% of correct responses. **c**, Performance has an initial quick phase of improvement within a few daily sessions, followed by a slower rate of improvement over a period of 10–15 sessions. The obtained level of improvement is almost entirely preserved for up to 22 months after the experiments were done. Adapted from ref. 38.

This degree of specificity also has important implications for the way in which acquired information is represented in the cortex. A suggested mechanism is referred to as 'cortical recruitment'. This involves an increase in the area of cortex representing the trained location. Experiments demonstrating this phenomenon were done in the somatosensory and auditory systems^{27,28}. However, even here other cortical changes seem to correlate better with the improvement in performance. These include a change in the temporal characteristics of neuronal responses, with a more reliable entrainment of the response to the periodicity of the stimulus²⁹. In the visual system no such cortical magnification has been observed²⁵. It is still unclear whether there are differences between results from the visual compared to other sensory systems, although there are some differences in the experimental designs used. For example, in the visual studies emergent properties of cortex are associated with training, whereas in the somatosensory and auditory systems properties of the cortical inputs are involved. Modelling the cortical changes underlying perceptual learning must allow for the specificity of learning for the trained stimulus. Moreover, these models must be consistent with the finding that training on one stimulus at one location does not produce a degradation in performance when discriminating other stimuli at other locations.

Time course of perceptual learning

An important component of models of perceptual learning is the rate at which learning occurs. As shown below, in some experiments there is an initial period of fast learning, which is then followed by a much slower rate of improvement (see Fig. 4). Several neural network models are able to reproduce this behaviour, albeit by using different mechanisms. In the feedforward network of Poggio *et al.*⁵ (Fig. 3), during the first phase of learning new units are added to the intermediate layer of the network, ensuring the coverage of all the space of possible input patterns. As a result, the classification error rate comes within 10% of its asymptotic value after just several examples. This is followed by a later, slower phase of learning during which the architecture of the network remains fixed but the parameters of the network slowly adapt to their optimal values. This leads to incremental improvement in performance. In neural terms, the first phase could correspond to the recruitment of neurons in intermediate levels of visual processing which would represent the stimuli encountered by observers at the beginning of practice. In Zhaoping *et al.*'s⁴ model (which combines both recurrent and feedforward mechanisms) two phases of learning could correspond to differing speeds of modification in the corresponding connections.

Perceptual learning requires repetition but not feedback

The improvement in performance seen in perceptual learning is proportional to the number of trials taken, although performance eventually asymptotes to a point beyond which additional trials make no further difference. During a discrimination task improvement is seen even in the absence of a reward or any indication that the correct response was made. Nevertheless, brain reward systems have been shown to have a role in perceptual learning. One of the sources of reward in the brain is thought to be the cholinergic input from the nucleus basalis. Pharmacological blockade of the cholinergic input can inhibit, and stimulation of the nucleus basalis can promote, perceptual learning^{30,31}. So, it is possible that mere performance of the task has an implicit reward associated with it, even when a reward is not given in every trial. Although learning can occur in the absence of feedback, feedback can facilitate learning. Moreover, feedback that is uncorrelated with the response disrupts learning. But the nature of effective feedback is interesting, because block feedback (that is, feedback after several trials, so in response to a certain percentage correct after a number of presentations) is as effective as trial-by-trial feedback⁶.

These observations put obvious constraints on the feedforward networks with supervised learning, in which feedback is usually

implemented as a 'teaching' signal that is required for the correct change in the strength of synaptic connections³². An interesting modification of supervised models of perceptual learning is that proposed by Herzog and Fahle³³. The main innovation of this model is that an internal evaluating feedback signal is used to guide selective connections between the input units of the model and the next network layer. Internal feedback is estimated as a difference between the responses of the output units to inputs that have to be discriminated. Learning then selectively inhibits the feedforward connections that are not providing the signal required to increase the evaluated performance (a process called gating; see refs 34, 35 for similar ideas). Unsupervised learning algorithms in feedforward networks have also been proposed^{36,37}.

Longevity of perceptual learning

A striking long-term stability of the improvement in performance is observed in certain tasks. For example, in Karni and Sagi's experiments on texture discrimination³⁸ subjects achieved a significant improvement in performance over four to five days. However, once subjects learned the task, they maintained their improved level of performance for at least three years without needing further practice. This observation particularly challenges any model that is based on activity-dependent synaptic plasticity in the sensory areas. Obviously, neurons in these areas are constantly responding to a continuous stream of sensory inputs that should, with time, wipe out specific traces produced by training. A possible explanation for long-term improvement could be that a certain fraction of synaptic connections becomes resilient to modification as a result of perceptual learning (see review in this issue by Abbott and Regehr, page 796). Alternatively, training on one task could affect a small subset of inputs that are only engaged when that task is performed. Even if the same cells participate in different tasks they may engage different inputs. This would minimize negative interference in the traces produced by training on the different tasks. Understanding the causes for the striking longevity of perceptual learning and its dependence on the parameters of practice protocols may be an important step towards elucidating the process of consolidation of long-term memories in general.

Perceptual learning involves top-down influences

In most instances of perceptual learning the subject must attend to the trained stimulus for improvement to occur^{39,2}, although some studies have suggested that learning can occur in the absence of attention^{40,41}. This is one form of evidence for the role of top-down influences in learning; that is, for the regulation of information-encoding in lower-order cortical areas by higher-order areas. The top-down signal may be carried by cortical feedback connections. A generally accepted view of pathways of connectivity between cortical areas is that of a hierarchy which starts from primary sensory cortex and proceeds up to the highest areas encoding the most complex information. For every forward connection in this pathway, however, there is a reverse or feedback connection. The role of cortical feedback is little understood, but increasing evidence for attentional influences at early stages suggests that feedback may be involved in transmitting this kind of cognitive control. The attentional signal may have a role both in the ongoing processing of sensory information and in the encoding of learned information. A recent study has shown that the top-down influence can be extremely specific to different discrimination tasks at the same visual location. In these experiments, neurons in V1 changed their functional properties according to the task being performed, and these properties were only present when the animal was performing the trained task²⁶. So, there is a long-term change in function associated with the period of training (which can take place over many weeks), and a short-term switching between different functional states as the subject shifts from one trained task to another. The same top-down influences or feedback circuits involved in regulating the encoding of learned information may also be involved in its recall.

An appealing hypothesis, from a theoretical point of view, assigns to the feedback influences the role of transmitting to primary cortical areas signals that reflect the expectations of the sensory inputs. These signals are based on the internal representation of the sources of these inputs⁴². The neurons in the lower areas then respond to the deviations of the actual sensory inputs from the predicted ones. For this predictive coding system to work well it has to learn the statistical regularities in the sensory environment. This kind of model has not yet been directly applied to perceptual learning, and no direct experimental evidence for the effect of internally generated expectations on the neural responses in the primary sensory areas is currently available. Although there is no evidence that early sensory areas respond to deviations — instead they carry information more fully related to the stimulus — their tuning is clearly modulated by top-down influences of attention, expectation and perceptual task. Models that incorporate top-down interactions for both encoding and recall will assume increasing importance as experimental results provide further evidence for these interactions.

Cortical representation associated with perceptual learning

There is considerable debate concerning which cortical areas represent the higher order properties associated with contextual influences, and which circuits carry these influences (including intrinsic circuits within individual cortical areas and feedback connections to those areas). Even so, it is becoming increasingly clear that many areas, including primary sensory cortex, show functional changes that reflect learned information. The notion that different cortical areas specialize in particular kinds of information will probably change, for several reasons. As supported by both psychophysical and fMRI studies, the strategies that the brain uses for object recognition change depending on the degree of familiarity the subject has with the object. Learning to identify an object is associated with global changes in the representation of information across the cortical sensory pathway (Sigman *et al.*, submitted). Moreover, the act of object recognition does not involve a single cortical area but an interaction between multiple cortical areas and between forward, intrinsic and feedback circuits^{43,26} (also Sigman *et al.*, submitted). This of course creates a formidable challenge in terms of creating models that can replicate the multiple levels at which information can be represented in the cortex.

Rules of plasticity

The most generally accepted view of plasticity at the synaptic level is that, with coincidence in the activation of the presynaptic terminal and the postsynaptic cells, the synapses involved become strengthened⁴⁴. This hebbian rule is dealt with in more detail elsewhere in this issue (see review in this issue by Abbott and Regehr, page 796). But this rule has profound implications at the systems level, although some experimental results suggest that this rule does not operate exclusively. A fundamental question is whether sensory systems are designed to pick out exceptions in the environment, or to identify common coincidences. Coincidences or correlations in the environment can be represented at the level of correlations in neuronal firing, which then leads to synaptic strengthening. Information theoretic considerations, on the other hand, suggest that sensory systems are designed to pick up exceptions or changes in the environment (for example, the presence of a surface edge as opposed to the continuation of a uniform surface). Similar to JPEG compression, this would suggest that to carry the maximum amount of information along a limited number of channels (the optic nerve, for example), the functional properties of neurons have to be as distinct from one another as possible. This requires 'decorrelation' of their function, which suggests the need for an 'anti-hebbian' rule of plasticity⁴⁵.

A particular form of synaptic plasticity combining hebbian and anti-hebbian rules in a way that is motivated by recent studies on spike-time-dependent synaptic plasticity^{46,47} was proposed by Adini

and colleagues³⁷. Here, the learning rule chosen guarantees the convergence of the synaptic strengths to an equilibrium level after repeated presentations of the stimulus. However, synaptic modification restarts when the stimulus changes. This property could account for the saturation of perceptual learning after repeated practice. The perceptual task that was studied — contrast discrimination — seems to be saturated when it is performed on a wide range of contrasts, but not when a single contrast, or a few contrast levels in a fixed order are presented (see refs 48, 49 for a recent debate on this issue). When the stimulus configuration is changed during practice by adding surrounding components, Adini *et al.*^{7,49} observed an improvement in performance that was to a large degree independent of uncertainty in the stimulus contrast, in accordance with the above-mentioned feature of the learning rule.

Neuronal changes associated with perceptual learning

Various experimental observations, and computational models, have revealed changes in functional properties at the neuronal level that are associated with perceptual learning. These include changes in the tuning of neurons to the trained attribute. Steepening of the slope of the tuning curve reduces the threshold at which neurons show significant differences in response, and therefore the threshold required for discrimination^{11,12} (although others have failed to find such a change⁵⁰). This in effect leads to a reduction in the number of neurons responding to the stimulus, contrary to the observed increase in response in the cortical area representing the trained stimulus. Changes have been observed in the timing and reliability of neuronal responses, which represents an increase in signal to noise. This also leads to a reduction in the threshold at which there are significant changes in response. Along with improvement in discrimination of more complex forms, neurons show changes in contextual tuning. This is the way in which the elements of a complex stimulus interact, in terms of neurons' responses, as the stimulus configuration is changed.

Outlook

Many facets of perceptual learning have been successfully reproduced in simple, but plausible, neural network models. These models provide important insights into possible mechanisms which can then be tested experimentally. But, so far, these models are far too specific to provide a full account of the phenomenology of perceptual learning. As a result, they do not support a more general understanding of the neuronal processes underlying early stages of information processing. What is needed is a combination of feedforward models with models based on lateral feedback and top-down influences representing the task, expectations, attention and signals controlling synaptic modification. Future work will have to increasingly include details of the biophysical mechanisms of synaptic learning in cortical networks. □

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Cortical rewiring and information storage

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Current thinking about long-term memory in the cortex is focused on changes in the strengths of connections between neurons. But ongoing structural plasticity in the adult brain, including synapse formation/elimination and remodelling of axons and dendrites, suggests that memory could also depend on learning-induced changes in the cortical 'wiring diagram'. Given that the cortex is sparsely connected, wiring plasticity could provide a substantial boost in storage capacity, although at a cost of more elaborate biological machinery and slower learning.

The human brain consists of 10^{11} neurons connected by 10^{15} synapses. This awesome network has a remarkable capacity to translate experiences into vast numbers of memories, some of which can last an entire lifetime. These long-term memories survive surgical anaesthesia and epileptic episodes, and thus must involve modifications of neural circuits¹, most likely at synapses^{2,3}.

What changes in synapses underlie memory storage? The focus of neural learning research has been on activity-dependent 'weight' changes between previously connected neurons. This mode of plasticity could involve either changes in the efficacies of existing synapses, or structural changes that lead to the addition or subtraction of synapses between previously connected pre- and postsynaptic units (Fig. 1). In either case, the network's connectivity matrix, or wiring diagram, is left unchanged. (The term 'unit' could correspond to an individual neuron, although other assignments are possible; see below.) In the weight-plasticity scenario, the storage capacity lies in the system's ability to increase and decrease the weights on existing connections as a means of encoding learned information⁴⁻⁶ (Box 1).

In addition to weight changes, learning could involve alterations to the wiring diagram, whereby previously unconnected units become connected and vice versa (Fig. 1). Unlike weight changes, wiring changes require structural plasticity. In this learning mode, the storage capacity lies in the system's flexibility to choose which presynaptic units provide input to each postsynaptic unit (Box 1).

Weight and wiring changes are not mutually exclusive (wiring plasticity can even be viewed as a special case of weight plasticity; Box 1), and experimental evidence suggests that neurons and their synapses might be engaged in both forms of learning. It is well accepted that synaptic efficacy can be modulated in a use-dependent manner to produce weight changes⁷. Similarly, structural changes that would be required to achieve wiring changes, including synaptogenesis and outgrowth of axons and dendrites, can occur in the adult brain⁸⁻¹⁴.

Despite the likely coexistence of these two forms of plasticity in the adult brain, biological¹⁸⁻¹⁹ and computational²⁰⁻²⁴ considerations demand that weight and wiring changes be distinguished from each other. In most areas of the brain, including the mammalian cerebral cortex, only a small fraction of all possible connections between neurons physically exist, even within a local area^{25,26,40}. In such sparse networks, a capacity to rewire could dramatically increase the number of functionally distinct circuits available to

encode learned information. On the other hand, the task of finding appropriate partnerships between pre- and postsynaptic units in a sparsely connected network is a hard combinatorial search problem, and could require a large number of slow, 'generate and test' operations^{21,22}. Whether the brain has evolved the machinery to cope with these 'algorithmic' challenges remains an open question.

In this review, we discuss the possible role of wiring changes in the encoding of learned information in the adult

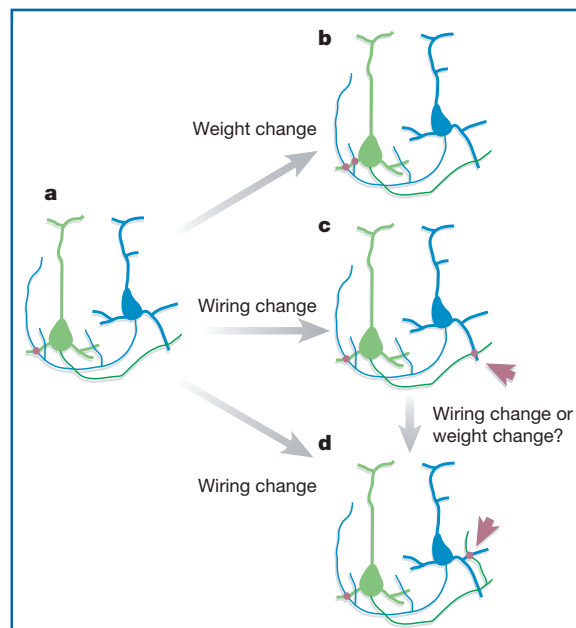
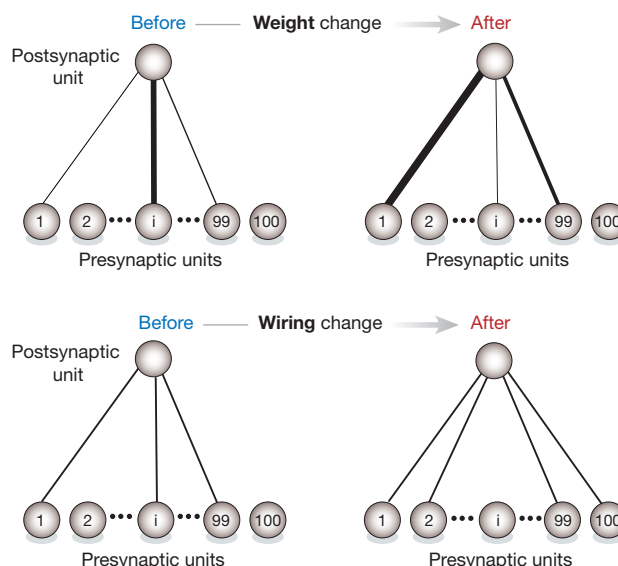


Figure 1 Structural circuit plasticity and the wiring diagram. The schematic shows two neurons (green, blue), dendrites (thick lines), axons (thin lines) and synapses (red circles). **a**, In the initial wiring diagram, signalling is from the blue neuron to the green one. **b–d**, Synapse formation and elimination can result in weight changes alone (**b**) or can include changes in the wiring diagram (**c, d**; red arrowheads point to changes). Wiring changes can occur with (**c**) or without (**d**) axon or dendrite growth. In the new wiring diagram, signalling occurs from blue to green and from green to blue. The transition between **c** and **d** might represent a wiring change, depending on the definition of the postsynaptic unit: the transition is a weight change if the postsynaptic unit is the whole neuron, and is a wiring change if the postsynaptic unit is a single dendritic branch.

Box 1

Contrasting weight versus wiring plasticity



The storage capacity of a neural network is a measure of the total learning-related flexibility of the circuit. If a network is represented as a graph of abstract units with weighted interconnections^{4–6}, then wiring changes are a special case of weight changes as they can be modelled by setting some non-zero weights to zero and vice versa. However, if the network is sparsely connected, it becomes useful to distinguish the two forms of plasticity.

Consider a postsynaptic unit with ten physical input connections ($s = 10$; for simplicity only three are shown above), and a population of $a = 100$ functionally distinct potential presynaptic partners. Each synaptic connection is assumed to have four possible long-term stable values (0, 1, 2 and 3; denoted by line thickness), giving $w = \log_2(4) = 2$ bits of storage capacity per connection. Assuming weight changes only, this system will have $s \cdot w = 20$ bits of total storage capacity, or two bits per synapse. However, if the cohort of axons connected to the postsynaptic unit can change over the

course of a learning episode, the capacity associated with this partnership choice is $\log_2(100 \text{ Choose } 10) = 46$ bits of storage, or 4.6 bits per synapse even with all weights held fixed. The larger the ratio a/s ; that is, the more axons that can serve as presynaptic partners for each postsynaptic site, the greater the in-principle advantage to a learning system that can choose its presynaptic partners.

The capacity advantage of wiring flexibility is greatest if/when individual synaptic weights can take on only a limited number of distinguishable values because of noise or other biological limitations. Some experiments hint at the possibility that synapses can have only a very limited range of long-term stable states^{57,58,89}, although this issue remains controversial and would benefit from further empirical study. Regardless of the value of w in any given neural circuit, it is reasonable to expect that the total learning-related storage capacity will benefit from both weight and wiring flexibility^{22,90}.

cortex. We discuss evidence and open questions relating to: (1) the identification of the presynaptic and postsynaptic units involved in learning; (2) geometric factors bearing on the inter-accessibility of axons and dendrites in the cortical microcircuit; (3) the existence of structural plasticity in the adult brain, including synapse formation and elimination, and outgrowth and retraction of dendrites and axons; (4) the stability of the neural circuit, that is, how long synaptic connections can be physically maintained; (5) the biological machinery that putatively manages learning-related cortical rewiring; and (6) interactions between weight plasticity and wiring plasticity.

What is a neural unit?

Identifying the neural substrate for learning and memory requires understanding which physical changes observed during learning lead to functionally distinct neural circuits. To do this, it is necessary to establish the proper mapping between the units and weights of the abstract network (Box 1), and the physical components of the biological neural circuit.

A unit is a node of the network whose state can be described by a single variable, such as a membrane potential, spike time or firing rate. In the cortex, one possibility is that individual neurons function as units, but this need not hold in general, and the mapping of presynaptic and postsynaptic units onto the neural hardware might be different.

A presynaptic unit might consist of the axon of a single neuron, or a group of functionally equivalent axons whose firing is strongly correlated. It is not known quantitatively how much overlap exists in the response properties of neurons within any given area of cortex, although there is evidence for substantial redundancy. For example, moving vertically through the layers of sensory cortex, neurons have heavily overlapping receptive fields, and even moving in the tangential direction, receptive field properties change gradually from neuron to neuron^{27,28}. This redundancy reduces the number of modifiable parameters available for learning, and thus works against capacity (although it might aid robustness). Estimates of the cell-to-cell redundancy for specific areas of cortex could be made using calcium or voltage imaging methods in behaving animals.

The issues involved in defining the postsynaptic unit are different. The goal is to identify the largest integrative unit whose modifiable parameters during learning consist of only the weights on each of its input connections. For example, the largest subdomain of a neuron whose overall integrative operation is linear would qualify as a postsynaptic unit. In contrast, any significant nonlinear spatial interactions between the inputs to a postsynaptic neuron would violate the above definition, and would force the adoption of a finer-grained mapping of units onto single neurons. Pyramidal neurons have most often been conceptualized as single integrative units, although over the past few decades, the idea that individual neurons could be

divided into functional subunits has had a steady presence in the modelling literature^{21,29–32}. Recent *in vitro* and modelling studies suggest that the integrative subunit of a cortical pyramidal cell might be as small as a single dendritic branch or less^{33,34}. Within certain limits, this reduction in the ‘grain size’ of the cortical network implies a larger number of postsynaptic units, and a greater overall storage capacity²².

Key questions remain unanswered, however. At present, we have no direct experimental evidence bearing on the number and size of integrative subunits within a pyramidal neuron *in vivo*. Subcellular functional imaging *in vivo*, perhaps using two-photon microscopy³⁵, could be used to map the receptive fields of individual dendritic branches and to help pin down the physical instantiations of presynaptic and postsynaptic units in the behaving brain.

How many wiring diagrams are within ‘immediate’ reach?

The storage capacity of a neural network depends in part on its ability to rewire, that is, on each postsynaptic unit’s flexibility to choose presynaptic partners from a larger set of candidates. This relates to the issue of sparseness as discussed in Box 1, and leads to two questions. First, how many axons representing different presynaptic units can connect to a given postsynaptic unit through spine/dendrite/axon outgrowth? Second, of those units that can potentially connect, how many actually do?

In answering these questions, it is convenient to distinguish two populations of possible synaptic partners, beginning with the population of synapses that can be formed without significant growth of axonal or dendritic arbors²³. This requires that an axon pass sufficiently close to a dendrite ($\sim 2\ \mu\text{m}$ or less) so that a newly formed dendritic spine or terminal bouton can bridge the gap between them (Fig. 2). Such points of apposition between dendrite and axon are called potential synapses²³. The number of potential synapses can be calculated from anatomical data using two different approaches. One is to calculate the expected number of axons passing within a spine’s

length of a dendrite. Such a calculation shows that potential synapses outnumber actual synapses by a factor of three to nine depending on the cortical area²³ (Fig. 3). However, this does not by itself imply short-range wiring flexibility; it must also be determined whether the population of axons within a spine’s length of the postsynaptic unit includes new potential partners, that is, presynaptic units that do not already form synapses elsewhere on the postsynaptic unit.

To help resolve this uncertainty, a second approach is to use reconstructions of axonal and dendritic arbors from a pair of neurons to calculate the expected number of potential synapses between them^{36–39}. Following this approach, it was determined that most neurons located within a few hundred micrometres of each other have at least one potential synapse between them. In other words, potential connectivity between neurons in a cortical column a few hundred micrometres in size is nearly all-to-all. This means that a connection between any two neurons belonging to the same cortical column can be realized by extending a spine or a terminal bouton. So, assuming that each axon carries a unique signal, and that each neuron is a single integrative unit, the storage capacity attributable to wiring plasticity within a cortical column can be substantial — $\log_2(\text{[number of neurons in column]}^2/\text{number of synapses in column}) = \log_2([10^7]^2/10^9) = 3\text{--}4$ bits per synapse — even if structural changes are limited to spines and synaptic terminals.

This estimate of capacity assumes that connected and unconnected local neurons contribute potential synapses proportionately, that is, the number of potential synapses between two neurons does not depend on the presence of an actual synapse between them³⁶. Electrophysiological measurements of synaptic connectivity between pairs of neurons, coupled with reconstructions of their axonal and dendritic arbors^{36,40}, could test this assumption. If the assumption is validated, many of the potential synapses considered above could belong to previously unconnected neurons, meaning that bona fide wiring changes could take place in cortical tissue with only minimal structural adjustments²³.

Evidence for synapse formation and elimination

As previously noted, synapse formation and elimination could contribute to changes in either weights or wiring. As such, simply observing synapse addition and subtraction does not help to distinguish between the two basic modes of plasticity, but would imply that wiring plasticity is at least mechanistically possible. Several types of experiments have provided evidence that synapse formation and elimination occurs in the adult brain. Electron microscopic analysis has provided evidence for new synapses in sensory cortex after behavioural enrichment⁸ and sensory stimulation⁹. Similarly, long-term, high-resolution imaging experiments in the somatosensory cortex have shown that some dendritic spines appear and disappear, and that the rate of turnover is modulated by sensory experience¹⁰. Subsequent electron microscopic analysis revealed that at least some of these new spines make synapses. Together these experiments provide convincing evidence that the adult brain maintains the capacity for synapse formation and elimination. *In vivo* imaging experiments have also revealed that a fraction of dendritic spines is stable over months, and this fraction might be higher in the visual than in the somatosensory cortex^{10,18}. It is even possible that a subpopulation of synapses persists for most of the life of the animal and that the fraction of stable synapses differs between different cortical areas.

How quickly can new spines form and how long do they, and their synapses, live under diverse experiential conditions? Is the cortical circuit structurally plastic at the level of spine changes, but built on a skeleton of stable dendrites and axons? Answers to these questions could come from time-lapse *in vivo* imaging to track the fates of synaptic structures, such as spines, axonal varicosities and labelled vesicle clusters. However, optical microscopy has certain limitations. High-resolution optical measurements are mostly limited to the superficial layers of the neocortex⁴¹ (but see ref. 42). Furthermore, optical techniques alone do not inform unambiguously about

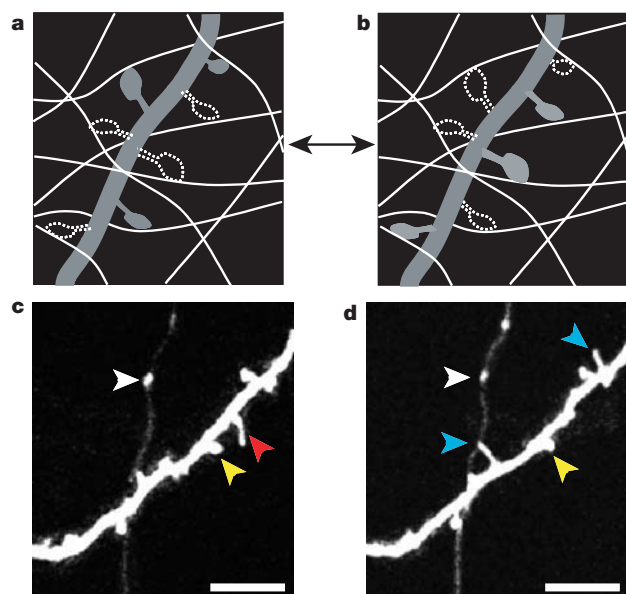


Figure 2 Structural plasticity. **a, b**, Schematic of structural plasticity with fixed potential connectivity. Only two of many possible configurations are shown. Dendrites and existing spines are grey. White lines denote axons, dashed white lines are potential synapses. **c, d**, *In vivo* microscopy of structural plasticity (A. Holtmaat, unpublished), showing a dendritic branch (thick line) and an axon (thin line). The picture in **d** was taken 16 days after the one in **c**. Note the appearance (blue arrow) and disappearance (red arrow) of dendritic spines. Some spines (for example, yellow arrow) and axonal terminals (for example, white arrow) are stable. Scale bar is $10\ \mu\text{m}$.

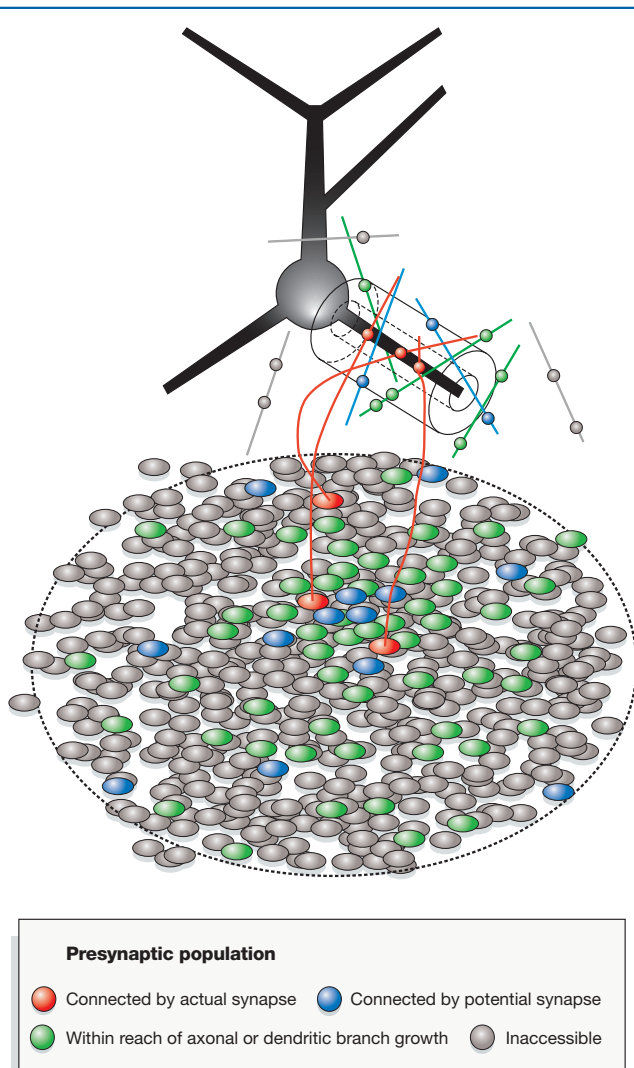


Figure 3 Actual and potential connectivity from a presynaptic population onto a postsynaptic unit. Concentric cylinders surrounding the postsynaptic dendrite show the volume accessible by the spine (inner cylinder), and the volume accessible by remodelling of an axon or dendrite (outer cylinder). Among those presynaptic axons that cross through the inner cylinder (blue), only a small fraction form actual connections (red). Green denotes the population of presynaptic candidates that cross through the outer cylinder. The much larger population of inaccessible axons is shown in grey.

synapse formation and elimination. Overlap of a dendrite and axon, or fluorescent labelling of presynaptic and postsynaptic components within an optical resolution element, do not necessarily imply the presence of a synapse there. Proof requires retrospective analysis using electronmicroscopy¹⁰, or perhaps physiological recordings with single synapse sensitivity⁴³.

Longer-range wiring connections

The second population of potential presynaptic partners consists of those that can be accessed only through growth of new axonal or dendritic branches. Their number depends on the maximum spatial extent of axonal and dendritic arbors, and can be estimated geometrically. Hypothetically, if axons and/or dendrites could grow without bound, all connections would be realizable. Then each synapse could encode $\log_2([\text{number of neurons}]^2/\text{number of synapses}) = \log_2([10^{11}]^2/10^{15}) = 23$ bits per synapse. Because physical constraints restrict the amount of biological wiring⁴⁴, the actual number is certainly far smaller (Fig. 3).

Evidence for dendritic growth in the adult brain

Do dendrites retain their ability to grow in the adult brain, and is such growth related to learning? Studies of dendritic plasticity make the reasonable assumption that synapses are formed and eliminated when dendrites grow and retract. Dendritic remodelling could therefore underlie rewiring of cortical circuits. The dendrites of cortical pyramidal cells can be visualized conveniently using the classic Golgi technique⁴⁵. Studies of dendritic plasticity have relied mostly on static measurements at a single time point and comparisons between groups of animals. A variety of experiential conditions have been tested, including the effects of environmental enrichment and behavioural training⁴⁵. Early studies focused on the effects of the complexity of the environment (for example, impoverished versus complex). With experimental manipulations beginning immediately after weaning, the structural differences are profound, on the order of 50% for higher-order dendrites¹¹. The effects of differential rearing on dendritic branching occur selectively in particular cortical areas (for example, the visual cortex, hippocampus), but not in other areas (frontal and motor cortex)^{46,47}. Dendrites have also been analysed after training in specific tasks in adult animals. For example, in one experiment rats were trained in a monocular task. Comparing dendritic arbors in the trained and untrained hemispheres revealed relatively subtle changes in the density of the most-distal branches of layer 4 and 5 neurons¹².

The static experimental design used in these studies of dendritic plasticity has obvious limitations: it is only sensitive to robust changes in the averages of morphometric parameters, and thus underestimates the dynamics and maximum spatial extent of the dendritic changes that have taken place in the course of learning. Furthermore, the use of the Golgi method complicates the interpretation of these studies. The method is capricious, and it is not known what determines which neurons are labelled or whether labelling of individual neurons is complete. Without this information, such experiments cannot be viewed as definitive. Recently, long-term, high-resolution *in vivo* imaging has become possible. Such longitudinal measurements are exquisitely sensitive, as they can detect dynamics without changes in averages. These experiments point to remarkable dendritic stability for periods of months in rodent primary sensory areas, including visual and somatosensory cortices and the olfactory bulb^{10,18,19}.

How plastic are dendritic arbors in the rest of the adult cortex? Is plasticity limited to particular parts of the dendritic arbor, to particular cell types, or to particular (for example, memory-related) cortical areas? Does it occur in response to learning, or only under conditions of chronic enrichment or deprivation? Long-term, time-lapse imaging *in vivo* could help to provide answers to these questions.

Evidence for axon remodelling in the adult brain

Cortical axons span many millimetres of cortical territory and target diverse areas. Long-range growth of cortical axons in the adult would therefore have profound implications for circuit plasticity and would probably imply rewiring. As for dendritic growth, axonal growth would imply changes in the complement of potential synapses.

Evidence for axonal growth comes from experiments involving lesions of the sensory periphery. For example, amputation of digits⁴⁸ or limbs⁴⁹ leads to massive reorganization of cortical circuits. In monkeys, physiological rewiring has been detected across long distances (>10 mm), suggesting large-scale cortical rewiring that could only be explained by axonal growth⁴⁹. Subsequent anatomical studies directly demonstrated growth of intracortical axons across several millimetres in the adult brain¹⁴. This process is of clinical importance because the extent of the rewiring correlates with the perception of phantom-limb pain⁵⁰.

Similar rewiring is observed in the primary visual cortex after focal retinal lesions^{13,51,52}. After several months, the cortical area corresponding to the retinal lesion becomes sensitive to surrounding regions of the visual world. This reorganization might be of value to

the animal because it could lead to perceptual fill-in and completion of visual contours. Direct anatomical analysis reveals that growth of horizontal axons could explain the functional changes triggered by retinal lesions.

These experiments reveal that cortical axons maintain the capacity to grow and elaborate in the adult brain. However, axonal remodelling has only been observed in response to prolonged (months to years) injury. In addition, such lesions are at least in some cases associated with massive subcortical changes, including transneuronal atrophy⁵³. Such pathological subcortical changes might release mechanisms of cortical rewiring that are not normally observed in the brain.

Clearly, our understanding of axonal plasticity in the adult brain remains in its infancy. How plastic are axonal arbors in the adult brain and what is the spatial range of growth? Do axons grow in response to learning, or only with injury? Just as for the question of dendrite outgrowth and remodelling, dynamic approaches using *in vivo* time-lapse imaging might help provide answers to these questions.

Finding good partnerships: an expensive proposition

It is clear that the adult cortex retains a substantial capacity for structural remodelling. However, a trade-off exists between the additional storage capacity made possible by long-range growth potential, in principle, and the additional space, time and biological machinery required to take advantage of it. First, the much larger presynaptic candidate pool accessible to a postsynaptic unit through long-range structural plasticity makes the search for groups of correlated afferents far more difficult. Second, longer-range connections presumably take longer to grow, forcing a slower learning rate. Third, longer 'wires' consume more space⁵⁴. As such, the spatial and temporal scales across which axons and dendrites can test and stabilize new connections could be important determinants of the learning rate and storage capacity of the adult cortex.

Setting aside the practical limitations on axonal and dendritic growth rates and tissue volume, the 'algorithmic' challenge faced by a structural learning rule is daunting in and of itself. To illustrate, we return to the example in Box 1, where the task facing the postsynaptic unit is to develop input connections from a particular set of ten axons, chosen from the 100 accessible axons in the neighbourhood. (In this example, we assume each axon represents a distinct presynaptic unit.) The basis for the postsynaptic unit's choice of presynaptic partners might be that the firing pattern of the to-be-selected group of ten axons expresses a statistically significant higher-order correlation; that is, the axons fire together more often than chance after normalizing for individual firing rates. Given that the postsynaptic unit has 17 trillion different combinations of ten axons to choose from, even in this small example, an efficient search scheme must be in place to pull out the special, correlated cohort of axons during the structural learning process. If there were no guidance mechanisms in place to support 'selection-at-a-distance', or for efficient triage of presynaptic candidates, the worst-case scenario could require that the postsynaptic unit sequentially, physically, 'interviews' all possible groups of ten candidate axons by first forming actual synaptic connections with them, and then testing their correlations through a postsynaptic signalling pathway. As should be evident from this example, an exhaustive physical search through the space of all accessible wiring diagrams is intractable.

Computer simulations of learning rules involving wiring plasticity confirm the need for a large number of generate-and-test operations^{21,22} — as are known to occur during development^{55,56} — but have also pointed to heuristics that can accelerate the learning process and boost storage capacity. In experiments with a structural rewiring learning rule²², it was found that when a new candidate synapse was brought in to replace a poorly performing synapse within a postsynaptic unit, the learning rate was accelerated and the final capacity was substantially increased, if at each iteration the new synapse was drawn from the top of a pre-screened candidate pool, rather than at random²². The pre-screened pool in the simulation experiments

could be analogous to the pool of 'silent' synapses (lacking AMPA receptors) that exists in pyramidal neurons^{57–59}. The physical convergence of a group of like-activated axons onto a compatible postsynaptic unit could also be accelerated through activity-dependent release of diffusible factors from axons and/or dendrites⁶⁰, or through electric fields⁶¹. Clearly, many open questions remain as to what biological mechanisms are needed, and which actually exist, to manage the search for new partnerships between unconnected presynaptic and postsynaptic units.

An additional question involves the rate and extent of synapse turnover that we should expect to see as learning progresses in a structure-based rewiring mode^{10,18}. Without a theoretical handle on this issue, we will not know whether, say, 1% synapse turnover per day is too little plasticity to be interesting, in that it signals that the system is virtually hardwired; whether it is too much plasticity to be interesting, in that virtually every plastic synapse will have turned over within a few weeks; or whether it is the optimal rate of turnover given the learning task at hand within the cortical area in question. Theoretical and modelling studies could help to shed more light on these questions.

Interdependence of weight and wiring changes

Although we have adopted the view that weight changes and wiring changes should be distinguished, it is nonetheless likely that if both modes of learning operate in the adult cortex, they will be mechanistically linked. In particular, the process of generating and testing new partnerships between presynaptic and postsynaptic units, a core operation in wiring plasticity mode, necessitates a hebbian LTP-like mechanism (see below) to stabilize newly formed connections when they correlate strongly with the overall postsynaptic response. Similarly, an LTD-like mechanism is required for the elimination of poorly correlated connections. This reflects the fact that at a very local level, the formation or deletion of a synaptic contact can simultaneously reflect a weight and a wiring change, with LTP and LTD as the bridging mechanisms. As a definitional matter, although the term LTP has been used to describe a wide variety of plasticity phenomena at diverse synapses with unknown mechanisms⁶², we use the terms LTP and LTD here to refer strictly to changes in synaptic efficacy at existing synapses. Candidate biological mechanisms for synaptic strength changes include modulation of the amount of neurotransmitter released per action potential, and the number and properties of synaptic glutamate receptors⁷.

Weight changes in the adult brain

What is the evidence for pure weight changes in adult learning? Detecting synaptic strength changes induced by experience-dependent plasticity⁶³ or learning⁶⁴ remains a great challenge. In the adult motor cortex, behavioural training can produce LTP-like potentiation of horizontal connections⁶⁵. However, in these experiments the synaptic mechanisms are not known and could involve structural, including wiring, changes. In the developing neocortex, deprivation-induced plasticity seems to be associated with changes in release probability⁶⁶ and changes in glutamate receptor number^{67,68}. However, plasticity in the developing neocortex also produces large-scale rearrangements of axonal⁶⁹ and dendritic arbors⁷⁰, and synapse formation and elimination⁷¹. It is therefore unlikely that changes in synaptic strength alone will comprise most of the circuit changes underlying experience-dependent plasticity in the developing brain.

Can the strengths of individual synapses be maintained for sufficiently long periods to explain long-term memory? A priori the answer to this question is uncertain because synapses often function with only a small number (about ten) of channels and receptors^{72,73}. Strength changes might therefore involve the modulation of only a few copies of proteins with short lifetimes⁷⁴. Long-term stability of synaptic strengths would then demand essentially single-molecule precision from the cell biological mechanisms that maintain

synapses. Information about synapse stability could come from long-term imaging of individual synapses *in vivo*. For example, imaging of synaptic receptors tagged with fluorescent proteins⁷⁵ over time would give an indication of the stability of synaptic structure and synaptic strength.

Causal relationship

Experiences that induce changes in synaptic function can also cause structural changes and wiring changes. This has led to the view that changes in synaptic efficacy, and synapse formation and elimination might not be exclusive, but might operate on distinct timescales. Modification of synaptic function could operate in seconds to hours, whereas structural changes become important over longer periods². This view is supported by studies of the gill-withdrawal reflex of *Aplysia*^{2,15}. Somewhat analogous results come from studies in cultured hippocampal brain slices, where stimuli that induce LTP also lead to the growth of dendritic spines^{76,77} that make new synapses⁷⁸. These new synapses appear delayed compared with synaptic potentiation, indicating that they could be part of a late phase of synaptic plasticity.

Shared cell biological and molecular mechanisms

Can molecular techniques help to distinguish between the roles for weight versus wiring changes in experience-dependent plasticity and in learning and memory? Several interdependencies could complicate the interpretation of molecular interventions. In the process of synapse formation, contact formation between dendrite and axon triggers the delivery of presynaptic release machinery and postsynaptic receptors to synapses⁷⁹. Maturation of synapses involves hebbian forms of synaptic plasticity^{80,81}. Consistent with this, LTP is especially pronounced during developmental periods of massive synapse formation⁸². The cell biology of synapse formation and elimination, and synaptic strength changes, therefore share cell biological mechanisms.

Shared molecular pathways also exist at the level of induction of plasticity. For example, one of the better-studied pathways involves the calcium/calmodulin-dependent protein kinase (CaMKII). CaMKII clearly has a prominent role in LTP: it is necessary for the induction of LTP and is activated persistently by stimuli that produce LTP. Moreover, activated CaMKII is sufficient to potentiate synaptic transmission. CaMKII also has a role in plasticity *in vivo*: genetic disruption of CaMKII function prevents experience-dependent plasticity of receptive fields and hippocampal-dependent learning. Does this mean that CaMKII and LTP are the molecular and cellular substrates of memory? The problem with this interpretation is that the CaMKII pathway is not specific to LTP. Rather, a large class of activity-dependent responses involve CaMKII signalling, including dendritic and axonal branching in the developing brain⁸³, the formation of spine synapses⁸⁴, and changes in the wiring diagram in cultured neurons⁸⁵. Genetic perturbations of CaMKII therefore probably interfere with both LTP and wiring plasticity. Experiments involving perturbations of other molecular pathways are similarly difficult to interpret in terms of circuit mechanisms.

An important question for future research is whether a core of molecular pathways exists that is specific to modulation of synaptic transmission as opposed to structural change. Given knowledge of such pathways, spatially and temporally precise molecular perturbations could yield important information on the role of structural plasticity and wiring change in the adult brain. However, even if such core pathways are identified, molecular perturbations could be difficult to interpret. Genetic perturbations of structural plasticity would presumably change the patterns of activity in neural circuits, which could change synaptic strength.

Future directions

We have argued that learning-related plasticity in the cortical wiring diagram, mediated by structural changes in spines, dendrites and axons, could underlie a second mode of long-term information storage

in the adult cortex that operates in addition to the more commonly accepted learning mode based on changes in synaptic weights. Proof that wiring changes have a major role in adult learning will depend on further developments in imaging technologies to allow subcellular visualization of neural activity and morphological changes in the brains of behaving adult animals. An alternative approach could involve the development of new technologies to allow rapid analysis of synaptic circuits on a large scale. This might include high-throughput serial-section electron microscopy to allow the reconstruction of the synaptic circuits defining entire cortical columns in individual animals. Data of this kind would allow comparison of cortical circuits in animals that have, and have not, undergone particular forms of training.

We have emphasized that a fuller understanding of the role of wiring plasticity in adult learning depends not just on gathering more and better data showing the dynamics, spatial extent and longevity of learning-related structural changes in the adult brain. It also depends on: (1) a fuller description of the integrative properties of individual cortical neurons; (2) better models of the representational redundancies that exist among the neurons within the cortical column; (3) better geometric models of pyramidal cell morphologies and of the spatial intercalation of axons and dendrites in the cortical neuropil; and (4) a more complete description of the guidance and triage mechanisms that, just as in early development, promote the gathering together of correlated axon terminals onto postsynaptic targets.

More global 'systems' issues ought to be considered as well. For example, given that the encoding of information through learning-induced wiring changes is an inherently slow process, we must consider what strategies the brain might have adopted to buffer the flow of incoming information while it is being (slowly) structurally encoded. The proposal that information is rapidly encoded in the hippocampus during episodic learning (weight plasticity?), and later consolidated in cortical tissue over many months (wiring plasticity?), is highly relevant to the present discussion^{86–88}. It may also be possible to search for congenital and/or disease-related long-term memory deficits that can be causally connected to the absence or dysfunction of factors contributing to structural plasticity and neurite guidance.

The identification of the engram — the physical change(s) encoding a particular long-term memory — remains a key aim of the field. In approaching this and other difficult questions relating to the physical substrate for long-term storage in the adult brain, an interdisciplinary approach that combines anatomical, physiological, molecular and theoretical methods seems the most likely to succeed. □

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Plasticity in single neuron and circuit computations

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Plasticity in neural circuits can result from alterations in synaptic strength or connectivity, as well as from changes in the excitability of the neurons themselves. To better understand the role of plasticity in the brain, we need to establish how brain circuits work and the kinds of computations that different circuit structures achieve. By linking theoretical and experimental studies, we are beginning to reveal the consequences of plasticity mechanisms for network dynamics, in both simple invertebrate circuits and the complex circuits of mammalian cerebral cortex.

The nervous system shows considerable plasticity, allowing animals to adapt to changing internal and external environments. During development, learning and in ongoing behaviour, individual neurons, synapses and the circuits they form show short-term and long-term changes as a result of experience. Plasticity occurs at all levels, from the behaviour of single ion channels to the morphology of neurons and large circuits and over timescales ranging from milliseconds to years. Because plasticity in the brain occurs at so many levels of organization and over so many timescales, theoretical and computational methods are required to understand how adaptive change to brain function and behaviour is brought about. Many studies of plasticity in the brain have focused on memory storage and retrieval. However, plasticity and neuromodulation also have crucial roles in altering excitability in the brain and regulating behavioural states, such as the transitions between sleep and wakeful activity. Theoretical work is also needed to understand the computational consequences of these various plasticity and modulation mechanisms. Here, we illustrate the use of combined theoretical and experimental approaches for understanding neuronal and circuit dynamics, using examples from both small invertebrate and large vertebrate circuits.

The building blocks of circuit plasticity

Neurons communicate with each other by means of chemical and electrical synapses. It is now clear that the strengths of many, if not most, synapses are altered by either the temporal pattern of firing of the presynaptic neuron and/or by amines or neuropeptides delivered hormonally or by neuromodulatory neurons¹. Some synapses show short-term depression in which the amplitude of successive synaptic potentials progressively decreases. Others show rapid facilitation in which successive synaptic potentials grow in amplitude. (For a detailed discussion of the computational potential of short-term plasticity of synaptic strength, see review in this issue by Abbott and Regehr, page 796.) Much attention has been paid to the computational consequences of long-term use-dependent changes in synaptic strength, such as that seen in long-term depression (LTD) and in long-term potentiation (LTP). It is also clear that the specific timing of activation of presynaptic and post-synaptic activity is crucial for the induction of plasticity^{2,3}. Synaptic strength can be modulated by amines and neuropeptides that act on presynaptic terminals to alter the amount of neurotransmitter released with each action potential⁴. Again, this can result in short-term or long-term

modifications of synaptic strength⁵, depending on how often the neuromodulator is applied.

Although historically most theoretical studies of memory storage in neural networks focused on changes in synaptic strength as the mechanism for implementing stable changes in network behaviour⁶, it is now evident that changes in the intrinsic firing properties of individual neurons also have important roles in altering circuit behaviour. Because some ion channels have slow kinetics, a neuron's response to a synaptic input can reflect the neuron's history of activation⁷. There are numerous use- and modulator-dependent alterations in channel number and distribution that can also influence a neuron's excitability and the way it responds to synaptic inputs^{8,9}. Changes in both synaptic strength and a neuron's intrinsic firing properties will alter circuit dynamics. This is illustrated in Fig. 1 where the dynamic clamp¹⁰ is used to construct a simple two-neuron circuit in which each neuron is inhibited by the other¹¹. The dynamic clamp is used to alter the strength of the synapses, or the amount of one of the membrane currents, I_H (hyperpolarization-activated inward current). Similar changes in the period of the circuit oscillation were produced by changes in both the synaptic and I_H conductances. This illustrates that it is impossible to a priori predict the mechanism that produces a change in network output, and that without theoretical methods, it is difficult to understand how the dynamics of even such small circuits depend on the properties of their underlying neurons and synapses. Much important theoretical work has been done on simplified and small circuits. But understanding how the functions of large circuits in the vertebrate brain are altered by plasticity demands an understanding of how to study those large circuits and how to evaluate and understand changes in their behaviour when synaptic and intrinsic properties are altered.

Structural complexity of neurons

Cajal¹² showed that individual neurons have extraordinarily complex anatomical forms that are characteristic of a given neuronal cell type. The beauty of these structures makes the implicit promise that they have meaning — a premise that was supported by the influential theoretical work of Rall on integration in passive cables^{13–15}. Using Rall's cable theory, it is possible to predict the attenuation of a given synaptic input as a function of its position in the (passive) dendritic tree. The emergence of visually-guided patch-clamp recording techniques has since made it possible to routinely record from dendrites, and to perform multi-site dendritic recordings in the same neuron. These techniques have

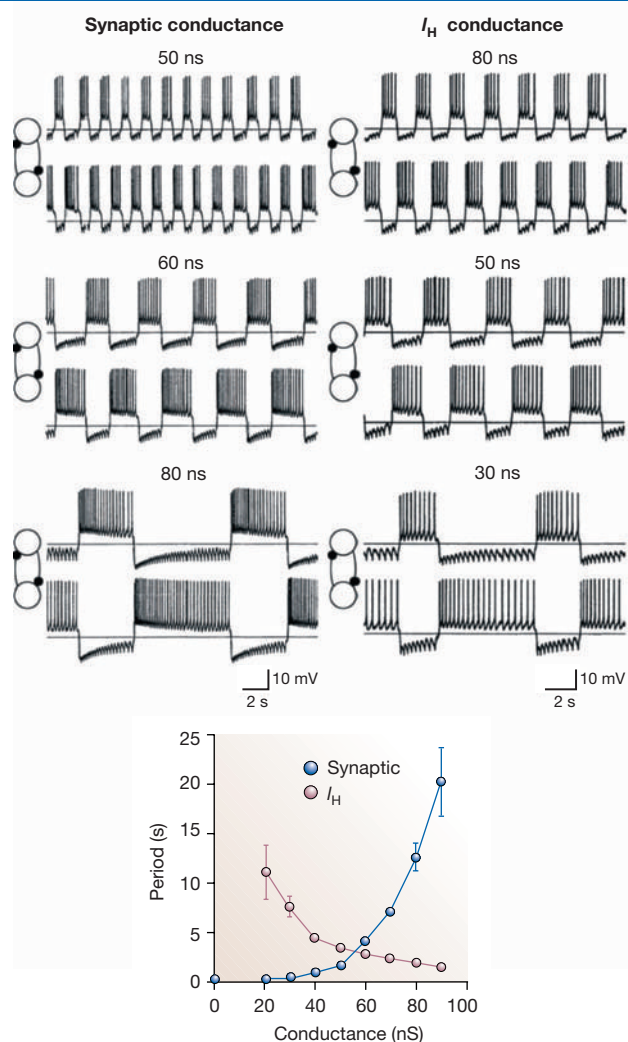


Figure 1 Plasticity of circuit dynamics can arise from modifications of synaptic strength or of intrinsic membrane currents. The dynamic clamp is a method that allows the investigator to add a programmed conductance to a biological neuron. In the example shown here, the dynamic clamp was used to create artificial reciprocal inhibitory synapses between two biological neurons that are not connected by biological synapses. Additionally, the dynamic clamp was used to add an I_H conductance to both neurons. Because the amount of the programmed conductances is under investigator control, the effect of altering the conductance on the network's output can easily be determined. Two biological neurons are synaptically coupled using the dynamic clamp. Modified from ref. 11.

revealed that dendrites contain many ion-channel types^{16–19}, and that they can produce Na^+ and Ca^{2+} spikes, which propagate towards the soma or away from it^{16,19}. The presence of dendritic ion channels may also modify the amplitude and shape of synaptic inputs^{20–22}, sometimes correcting for dendritic filtering, or have more subtle effects like establishing coincidence detection^{23,24}. The emergence of efficient techniques to perform three-dimensional morphological reconstructions of single neurons, and of sophisticated numerical tools for simulating these morphologies^{25–27} now makes it relatively easy to develop semi-realistic computational models of the complex dendritic structure of neurons²⁶. As these computational models become standard tools in the laboratory^{25,27}, they will increasingly aid our understanding of how changes in the distribution and number of ion channels over the dendritic tree change the firing properties of neurons and their responses to synaptic inputs.

Dendritic action potentials probably have a central role in synaptic plasticity because they provide the strong depolarization necessary to establish coincidence of presynaptic and postsynaptic activity, which is required for inducing synaptic changes^{23,24}. Interestingly, this coincidence can be established by local dendritic spikes, without participation of the soma, which raises the possibility that local dendritic computations, or associations, can occur without participation of the cell body²⁸. These problems are now being heavily investigated; experiments and models are needed to explore the possible computations performed by the exquisite dendritic morphologies initially described by Cajal¹².

Regulation of intrinsic properties

A growing body of both theoretical and experimental work argues that part of a neuron's characteristic identity is a 'set-point' or target activity level that regulates the neuron's long-term mean activity level^{8,9,29,30}. In the intact and functioning brain, when neurons are receiving and responding to synaptic inputs, homeostatic maintenance of a neuron's activity level could be achieved by a global regulation of the strength of all of its synapses (synaptic scaling)³¹, by regulation of the excitability of the neuron itself^{9,32}, or by both. When neurons, or the circuits in which they reside, are silenced for one or more days, individual neurons respond by altering the densities of one or more ion channels³². Long-term compensation for changes in channel density or synaptic drive may require many of the same mechanisms that are used to produce changes in synaptic strength⁸. Moreover, because similar patterns of neuronal activity can be produced by various combinations of channel densities³³, it is likely that compensations for altered patterns of channel expression³⁴ occur frequently. Use-dependent alterations in conductance densities can occur on timescales ranging from minutes to hours^{8,35}, and so can compensate and be coordinated with similar timescale changes in synaptic efficacy.

Defining circuits

Neurons are connected into circuits by excitatory, inhibitory and electrical synapses that show a variety of amplitudes, time courses and time-dependent changes in synaptic strength. How then do we study the circuits underlying behaviour, and how do we determine how changes in circuit output depend on altered synaptic and intrinsic membrane properties? These problems have been approached differently for small and large circuits. In all cases it has become clear that computational approaches are needed to understand how circuit output depends on the properties of its components and their interactions.

The premise underlying the study of small invertebrate circuits was that it would be possible to: (1) characterize a behaviour; (2) identify the neurons participating in the circuit that produce that behaviour; (3) determine the connectivity among those neurons; and (4) understand how those neurons and their connections give rise to the behaviour. Towards this end, a number of invertebrate preparations were developed in the 1960s and 1970s. One of the hopes, perhaps naive, of these early workers was that similar circuit designs would underlie similar behaviour. As the circuits underlying a number of invertebrate central-pattern generators were described³⁶, it became clear that similar motor patterns could be generated by different circuit architectures and underlying cellular mechanisms. Nonetheless, it was possible to describe circuit 'building blocks' that are generally found to contribute to circuit dynamics in specific ways³⁷. For example, reciprocal inhibition (Fig. 1) is found in many motor circuits, where it often ensures that functional antagonists, such as extensor and flexor motor neurons, fire out of phase. This example illustrates the importance of theory: in the work on motor circuits, reciprocal inhibition is almost universally found to ensure alternation of firing between the neurons³⁸. Nonetheless, theoretical work showed that, depending on the time course of the inhibition, reciprocal inhibition can also

support in-phase firing^{39,40} — an insight that may be important in cortical dynamics⁴¹. This highlights the dangers of extrapolating the circuit consequences of even simple circuit configurations without fully understanding how circuit dynamics depend on the parameters of the underlying circuit elements.

Lessons from small circuits

A great deal is now known about how the small circuits that generate rhythmic behaviour in invertebrates are organized and about how they function^{42,43}. This is because it is relatively easy to determine which neurons are 'part of the circuit' and to identify how they are connected as these circuits have easily measurable and definable outputs. Sensory and motor circuits can easily be studied in relation to sensory stimuli or to motor behaviour, but defining circuits becomes more nebulous as we move further to the higher centres in the brain where cognitive processes take place. That said, what has been learned from studies of small circuits and their plasticity that generalizes to larger and more complex circuits in higher animals and humans?

(1) Alterations in circuit function are often achieved by modifications of both intrinsic and synaptic properties. For example, in the pyloric rhythm of the lobster stomatogastric ganglion, the neuromodulator dopamine influences the strength of many of the inhibitory synapses within the network, and modifies I_A (the transient outward K^+ current) and I_H (ref. 44) in several network neurons. In the classic work on the gill and siphon withdrawal reflex in *Aplysia*, changes in both neuronal excitability and synaptic strength are produced by serotonin and experience⁴.

(2) Neuromodulation is the rule, not the exception. Individual neurons and individual synapses are often modulated by several substances, and many neuromodulatory neurons release a mixture of several cotransmitters⁴³. As the neuromodulatory environment changes, so will many properties of the cells and synapses that influence circuit function. As some circuit elements themselves contain neuromodulators, when these neurons are active, their released modulators will alter the circuit's dynamics⁴⁵. Consequently, as a circuit functions, this will itself alter the properties of its components.

In summary, the temporal dynamics and neuromodulatory environment specify the properties of the circuit which produces a specific output pattern. Changes in the neuromodulatory environment and changes in the circuit's own activity can in turn produce changes in output, and these changes contribute to behavioural plasticity on numerous timescales. However, to measure the properties of a single synapse, it is often necessary to silence the preparation so that the synapse can be studied in isolation. Likewise, to study the properties of a single neuron, it is customary to isolate it from its synaptic inputs. These two commonly implemented procedures mean that almost all measurements of synapses and cell properties are made under conditions that do not pertain during normal circuit operation. Therefore, it is desirable to use techniques such as the dynamic clamp¹⁰ and other modelling techniques to determine how circuit behaviour is likely to depend on the properties of the circuit elements.

Vertebrate circuits

Many of the principles first established from work on small circuits in invertebrates hold for the larger circuits in the vertebrate nervous system, in particular in those regions of the mammalian nervous system where the structure is relatively simple and the repertoire of intrinsic excitability well characterized. This is the case for structures such as the spinal cord, the inferior olive, the cerebellum, or the thalamus. Taking the thalamus as an example, thalamic cell types, their excitability properties and their connectivity are well defined⁴⁶. Thalamic neurons are endowed with complex intrinsic firing properties, such as rebound bursts, and they interact through many synaptic receptor types to generate oscillatory behaviour⁴⁷. Thalamic circuits are also subject to neuromodulatory influences⁴⁶. Acetylcholine, norepinephrine or serotonin affect intrinsic currents (Fig. 2a) and switch the circuit from an oscillatory mode to a 'relay mode'

in which oscillations are abolished. When these neuromodulators are present in activated states, they promote the relay of sensory information by the thalamus: their diminished concentrations during slow-wave sleep promote large-scale synchronized oscillations in the entire thalamocortical system.

For larger-scale circuits, such as in cerebral cortex, there has been no clear-cut identification of circuit behaviour. The cortical regions most accessible for study are those that are closely connected to the external world, such as primary sensory cortices or motor cortex. The primary visual cortex is characterized by the functional specialization of populations of neurons that respond to selective features of the visual scene. Cellular responses typically form functional maps that are superimposed on the cortical surface. V1 cortical neurons seem to obey well-defined rules of connectivity across layers, and make synaptic inputs that are well characterized and typical for each

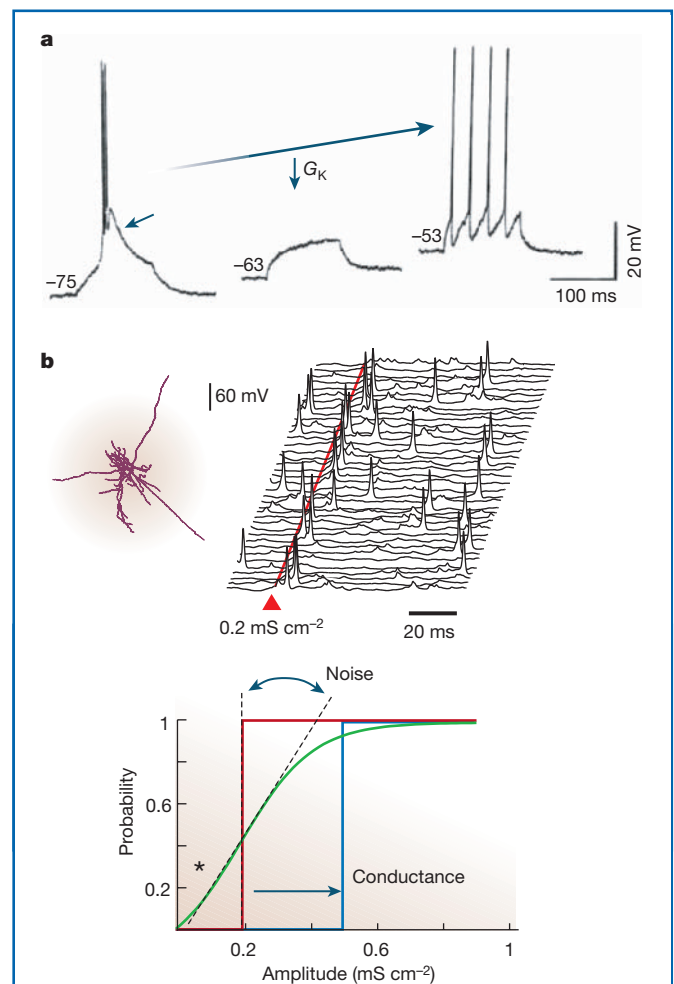


Figure 2 Different types of modulation of neuronal responsiveness. **a**, Neuromodulatory synapses that use transmitters, such as acetylcholine, norepinephrine or serotonin, can change the intrinsic excitability of the neuron. In the example shown here the neuromodulator acts to decrease a K^+ conductance (G_K), leading to an increase in excitability, and a switch from burst firing to tonic firing. Modified from ref. 48. **b**, Synaptic noise may have drastic effects on cellular responsiveness. This is illustrated here using a computational model of pyramidal neurons (upper left) in which synaptic noise is simulated by the random release of thousands of excitatory and inhibitory synapses distributed in soma and dendrites. A subthreshold input in quiescent conditions causes a well-detected response (upper right) in the presence of synaptic noise (red line; 40 trials shown). The response curve of the neuron is shown (lower panel) in quiescent conditions (red), with synaptic noise (green) and with an equivalent static conductance (blue). Synaptic noise changes the gain of neurons (slope of the response curve) and enhances the responsiveness to low-amplitude inputs (*). Modified from ref. 55.

layer. These data suggest a well-constrained wiring diagram across layers, and has motivated the concept of ‘cortical column’^{49–52}. According to this concept, there is a basic canonical pattern of cortical connectivity. In this scheme all areas of neocortex would perform similar computational operations with their inputs⁵³. However, even for the primary sensory cortices, there is no clear paradigm in which the distributed activity of neurons, their properties and connectivity have been characterized in sufficient detail to allow us to relate structure and function directly (as is the case for oscillations in small invertebrate preparations or in the thalamus). Nevertheless, using computational models, one can predict generic computations that cortical circuits could perform, a few of which are mentioned below.

One of the most striking differences between cerebral cortex and invertebrate networks is that cortical neurons *in vivo* show a considerable degree of apparent randomness in their activity. The membrane potential of cortical neurons shows fluctuating activity, mostly of synaptic origin, which is consistent with the extraordinarily dense connectivity in cortex⁵⁴. This ‘synaptic noise’ sets the membrane in a ‘high-conductance state’, which may affect the integrative properties of cortical neurons⁵⁵. Because studying dendritic integration *in vivo* is technically difficult, computational models are needed to reconstruct *in-vivo*-like conditions and to evaluate the impact of this synaptic noise on integrative properties. Such models predict that high-conductance states confer several computational advantages to cortical neurons⁵⁵. First, synaptic noise may boost the response to synaptic inputs⁵⁶ (Fig. 2b), in a similar way to stochastic resonance phenomena⁵⁷. This property was confirmed experimentally using dynamic clamp^{58,59}. Second, synaptic noise may reduce the dependence of the efficacy of synaptic inputs on their location in dendrites⁶⁰, resulting in a more ‘democratic’ dendritic tree in which each synapse exerts a similar vote in firing an action potential in the axon. This is, however, only valid for isolated inputs: the integration of multiple inputs may reveal the existence of ‘dendritic subunits’, as has been suggested by experiments⁶¹ and models^{62,63}. Third, synaptic noise sharpens temporal resolution, allowing cortical neurons to detect coincidences separated by milliseconds, and therefore to resolve precisely timed inputs^{55,64}. Finally, an obvious consequence of synaptic noise is that cortical neurons show a high trial-to-trial variability in their responses (Fig. 2b) — a feature often seen *in vivo*⁶⁵. Consequently, the only sensible measures that can be used to characterize the activity of a cortical neuron *in vivo* are probabilities. Indeed, probabilities have been used for decades to characterize responses recorded in cortex *in vivo*, under the form of ‘post-stimulus time histograms’⁶⁶. There is also a whole family of computational models of cortical coding based on probabilistic models⁶⁷, some of which are mentioned below.

Cortical computations

One of the most influential theories of neural computation was proposed by Hopfield⁶⁸, who showed that memories can be stored as stationary states (point attractors) in networks of simplified neurons. One advantage of this model is that it is mathematically similar to well-studied physical systems, and memory storage can be understood from the formation of minima in the energy landscape of the system. In these models, a hebbian-type learning rule (Box 1) can be used for modifying synaptic weights, and memories are distributed among the synaptic weights. However, the drawback of Hopfield’s theory is that there are no point-attractors in real networks of neurons, so its direct heuristic value in explaining cortical computations is limited. Nevertheless, this theory had the considerable merit of motivating generations of researchers to study computational models in neuroscience using the tools of mathematics and physics.

One generic computation of cortical networks may be to detect and extract correlations. Sensory systems must make sense of complex flows of information, in which exactly the same pattern is unlikely to happen twice. According to Barlow⁵³, the main task of our sensory

Box 1

Definition of hebbian and anti-hebbian rules (from ref. 100)

Hebbian rule

A given link will be strengthened (either by an increase of excitatory gain, or by a decrease of inhibitory gain) if the two units that it connects are active simultaneously.

Anti-hebbian rule

A given link will be weakened (either by a reduction of excitatory gain, or by an increase of inhibitory gain) if the two units that it connects are active simultaneously.

system is to detect (and model) correlations; it acts like a detective and notes, in the form of neuron firing, ‘suspicious coincidences’ in complex incoming information. It is these coincidences or correlations that may form the ‘objects’ or ‘features’ of our symbolic representations. After being detected by primary sensory areas, such correlations can be used for binding elementary features into more elaborate percepts. This binding problem has been intensely debated (for a recent review see ref. 69), and is based on the concept of neuronal assemblies, which are usually defined as a group of neurons that transiently undergo synchronous firing^{70–72}. This transient synchrony could form the basis of a common input to later stages of integration, and so promote responses that are specific to a given ensemble of features⁷¹. Thus, correlated firing serves here to form assemblies of neurons that are specific to a given feature. Cortical neurons should therefore be very efficient at detecting correlations⁷², as is indicated by computational models⁷³.

Another view, not necessarily contradictory, is that the cortex attempts to remove correlations. Probabilistic models have been proposed based on the observation that the cortex must infer properties from a highly variable and uncertain environment, and an efficient way to do so is to compute probabilities. One of the earliest probabilistic models proposed that the cortex infers probabilities based on ‘decorrelation’ or ‘redundancy-reduction’ operations^{53,74,75}. The most salient functional consequence of this is that these probabilities could be used to build efficient novelty detectors — a feature essential for survival. This redundancy-reduction function is also supported by the fact that the sensory system of mammals receives signals from millions of peripheral receptors sampling different features of the external world. Because many receptors convey similar information, the sensory system may need to reduce this redundancy to focus on the interesting aspects of the scene. This paradigm is particularly relevant to the retina, where the number of output fibres are two orders of magnitude less than the number of photoreceptors. Indeed, experiments provide evidence for redundancy reduction in this system⁷⁶.

The same ideas have been proposed for central structures such as the cortex. Here, an efficient way to reduce redundancy is to use synaptic interactions that obey the anti-hebbian rule (see Box 1). This type of plasticity has been identified in synapses from parallel fibres on Purkinje cells in cerebellum⁷⁷, and in excitatory synapses between parallel fibres and medium ganglionic cells in the electrosensory lobe in electric fish⁷⁸. Networks with hebbian feedforward synapses combined with anti-hebbian recurrent inhibitory synapses were shown to efficiently decorrelate inputs, and they perform well in various unsupervised learning paradigms⁷⁹. Interestingly, several mechanisms present in cortical circuits can also have similar roles, such as spike frequency adaptation⁸⁰ or short-term synaptic depression⁸¹. Adaptation or plasticity processes remove correlations most efficiently over timescales comparable to their own characteristic relaxation time constant⁸⁰. This suggests that a broad range of dynamic processes is needed to cover the relevant timescales over which signals must be decorrelated. This is consistent with the fact that several mechanisms, possibly present in neocortex, such as

intrinsic adaptation, short-term synaptic depression, anti-hebbian plasticity, or even long-term changes of intrinsic properties, might have equivalent functional roles but complement each other at different timescales.

However, it is not clear that these ideas apply so straightforwardly to cortex, for several reasons. First, anti-hebbian plasticity has not yet been demonstrated in cortical recurrent connections, although it may be that plasticity of inhibitory connections has a similar functional role (see below). Second, in contrast to the retina, the number of cortical neurons, as well as the number of efferent axons, largely exceeds the number of ascending 'input' fibres⁸². There is, therefore, no structural constraint, as there is in the retina, which would call for redundancy reduction in cortex. Morphological and physiological data are more consistent with 'sparse codes' in which many units are used for coding, but extremely few units are active simultaneously^{79,83–85}. Third, other mechanisms also present in neocortex, such as hebbian plasticity^{86,87} or short-term synaptic facilitation⁸⁸, have the opposite role of enhancing pre-existing correlations⁸⁹ (Fig. 3). Thus, the cortex possesses mechanisms that are compatible with either reducing or enhancing correlations, and it is unclear whether these mechanisms coexist or whether they are expressed differentially according to context or cortical area. Neocortical circuits dominated by anti-hebbian and depressing mechanisms may serve as novelty detectors by decorrelating afferent inputs and therefore function in a 'search mode'. This mode would be a priori compatible with primary sensory areas. However, other cortical circuits, dominated by hebbian and facilitating mechanisms, might function in a 'convergence mode', compatible with the type of operation performed in association or motor areas. It is not clear, however, whether these modes are separate or whether they coexist everywhere in cortex. In the latter case, any neocortical area would be equipped to function in both modes simultaneously or to switch between these modes depending on activity levels or neuromodulation.

Rather than attempting to explain cortical function on the basis of generic cellular and synaptic properties or stereotyped circuits, the diversity of cortical neurons and their highly complex synaptic connectivity can be used to propose a different computational paradigm. Cortical neurons show a wide diversity of intrinsic properties⁹⁰. Likewise, synaptic dynamics are richly variable and show properties that range from those of facilitating to depressing synapses⁸⁸. Indeed, the essential feature of cortical anatomy may be that there is no canonical pattern of connectivity, consistent with the considerable apparent random component of cortical connectivity templates^{54,91}. Taking these observations together, the cortex may be seen as a circuit that maximizes its own complexity, both at the single-cell level and at the level of its connectivity. In support of this view, computational models are now emerging in which the goal is to take advantage of the special information processing capabilities, and memory, of such a complex system. Such large-scale networks can transform temporal codes into spatial codes by self-organization⁹², and computing frameworks have been proposed which exploit the capacity of such complex networks to cope with complex input streams⁹³ (Fig. 4). In these examples, information is stored in the ongoing activity of the network, in addition to its synaptic weights. A given output can be provided at any time within this ongoing activity, rather than requiring the system to converge towards predefined attractors. The concept of the cortex as a 'large network of identical units' should be replaced with the idea that the cortex consists of 'large networks of diverse elements', where cellular and synaptic diversity are important for computation.

Towards understanding the many facets of plasticity

Several issues must be considered when linking plasticity mechanisms with neuronal computations. First, the rules that govern the plasticity at many inhibitory synapses are unknown. One possibility is that the inhibitory feedback from local interneurons obeys anti-hebbian plasticity, which would be consistent with the predictions of models of redundancy reduction. In contrast to the very large number

of studies modelling memory storage in networks using changes in excitatory synapses, few models implement learning rules for inhibitory synapses. Nonetheless, recent work showing that the balance of inhibition and excitation can be important for gain modulation^{56,58}, and in the genesis of functional selectivity⁹⁴, illustrates the importance of determining the rules that control the strength of inhibitory synapses.

Second, plasticity mechanisms are likely to depend on behavioural state, such as deep sleep or aroused states. Most experimental studies of the mechanisms underlying synaptic plasticity have been done in slices or in anesthetized preparations. However, these preparations differ from aroused and attentive animals, during which cortical networks are in high-conductance states⁵⁵, maintained by the release of a number of neuromodulators, such as acetylcholine and norepinephrine⁹⁵. These substances may considerably affect the plasticity mechanisms of cortical circuits^{96,97}. It is, therefore, imperative to verify that the plasticity mechanisms found in slices apply to the

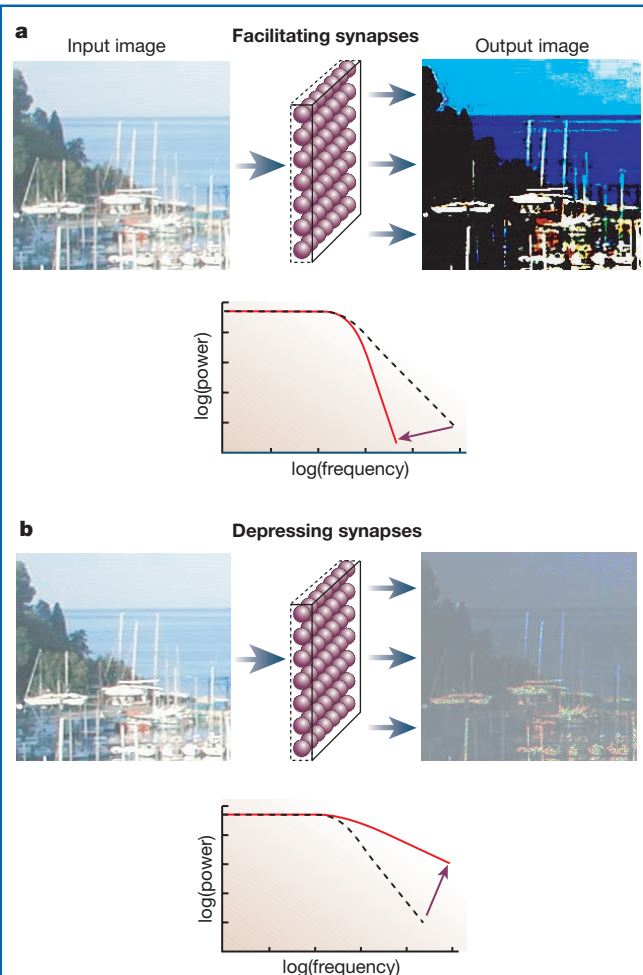


Figure 3 The type of transformations realized by synaptic plasticity. **a**, Facilitating synapses enhance existing correlations. When an image, such as the natural scene shown here, is processed by a neural network with facilitating synapses, correlations are reinforced, or equivalently, the spatial power spectrum is more structured (see graph). The result is an output image which has enhanced contrast. Enhancement of correlations can also be obtained using hebbian synaptic plasticity. **b**, Similar model with depressing synapses. Here, the transformation results from reducing correlations, or equivalently, reducing redundancy. This redundancy reduction corresponds to whitening the spatial spectrum of the image (see graph). The reduction of existing correlations leads to an output image in which many details are lost. A decorrelation can also be obtained using anti-hebbian synapses or adaptation mechanisms.

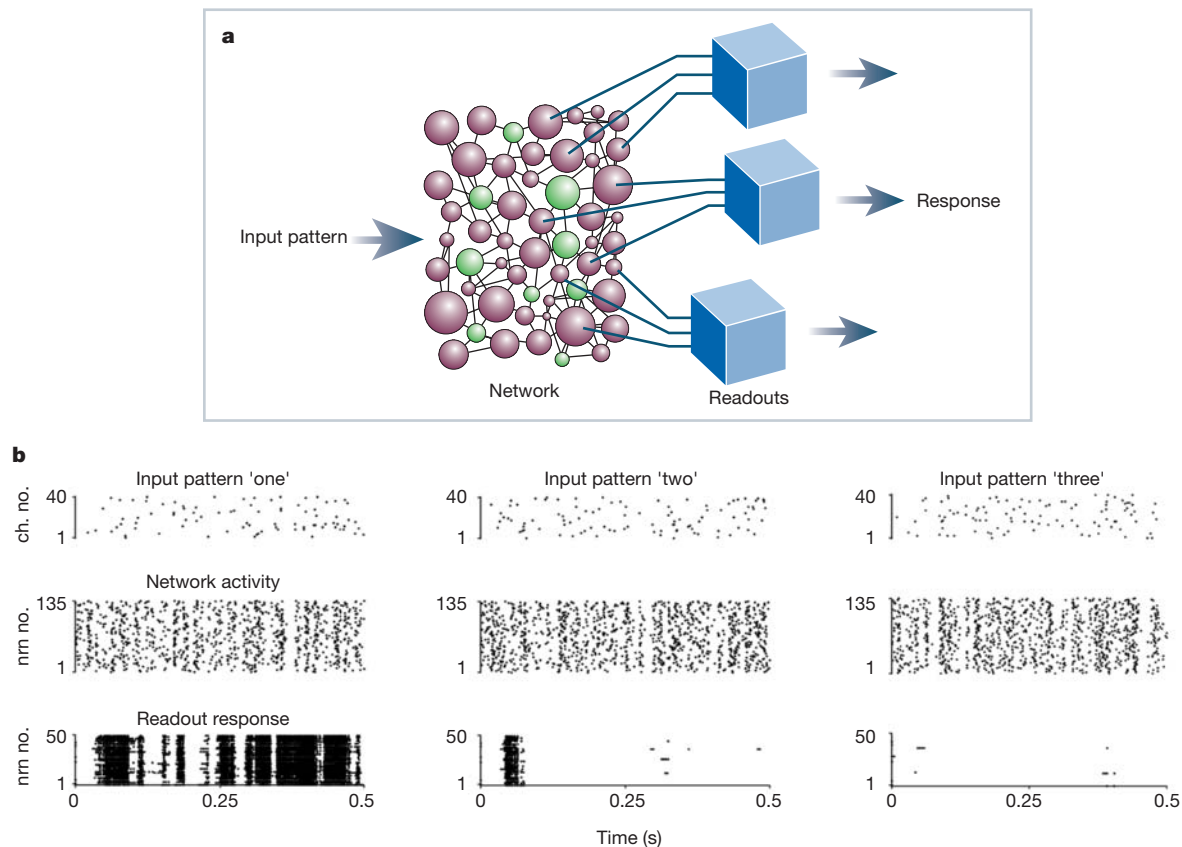


Figure 4 Computing with network complexity. **a**, Scheme of a computational model that uses a network in which diverse cell types and synaptic interactions are taken into account. The activity of a few cells are fed into 'readouts' (blue), which extract the response from the complex dynamics of the network.

b, Example of computation of different spoken words. The ongoing network activity is apparently random and similar in each case, but it contains information about the input, which can be retrieved by the readout. Modified from ref. 93.

activated brain. The relative ease of inducing and consolidating plasticity in slices may also indicate that these mechanisms are best expressed during states of low release of neuromodulators, such as during slow-wave sleep. This would corroborate recent evidence that slow-wave sleep is actively implicated in the consolidation of memory traces⁹⁸, and models of learning that require a 'sleep' phase⁹⁹. Consistent with this idea, the widely synchronized oscillations characteristic of slow-wave sleep are likely to constitute an optimal signal for inducing plastic changes in the network⁴⁷. Relating plasticity mechanisms to the state of the network constitutes an essential piece of information that should be targeted by appropriate experiments and theories.

Outlook

How far have we come in understanding how neuronal circuits produce behaviour? Certainly, considerable progress has been made for some relatively simple, small circuits^{4,42,43,45}. These small circuits provide ideal platforms for understanding which circuit parameters are genetically specified, and how circuit properties are modified by experience. A more daunting challenge is to link circuitry with behaviour for more complex networks, such as cerebral cortex, because the computational operations in cortex are still largely unknown. It is clear that cortical neurons possess complex intrinsic properties and that their rich and diverse synaptic connections are subject to plasticity, modulation and noise over many timescales. Many of the concepts arising from studies of small networks may extrapolate directly to the cortex, and our present inability to understand cortical function could be just a matter of complexity arising from its large size and multiple cell types. If so, we need to develop

appropriate conceptual, physiological and computational tools to handle this complexity. Alternatively, we may be missing a fundamental 'building block' that is required to understand cortical function. In either case, there is presently no coherent theory of cortical computations, and constructing one will be possible only through a tight combination of experimental and theoretical approaches. □

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Synaptic computation

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Neurons are often considered to be the computational engines of the brain, with synapses acting solely as conveyers of information. But the diverse types of synaptic plasticity and the range of timescales over which they operate suggest that synapses have a more active role in information processing. Long-term changes in the transmission properties of synapses provide a physiological substrate for learning and memory, whereas short-term changes support a variety of computations. By expressing several forms of synaptic plasticity, a single neuron can convey an array of different signals to the neural circuit in which it operates.

Synapses conduct signals between neurons in an ever-changing manner. The effect of a signal transmitted synaptically from one neuron to another can vary enormously, depending on the recent history of activity at either or both sides of the synapse, and such variations can last from milliseconds to months. Activity-dependent changes in synaptic transmission arise from a large number of mechanisms known collectively as synaptic plasticity. Synaptic plasticity can be divided into three broad categories: (1) long-term plasticity, involving changes that last for hours or longer, is thought to underpin learning and memory^{1–3}; (2) homeostatic plasticity of both synapses and neurons allows neural circuits to maintain appropriate levels of excitability and connectivity despite changes brought about by protein turnover and experience-dependent plasticity^{4–6}; (3) short-term plasticity, which is the main focus of this review, occurs over milliseconds to minutes⁷ and allows synapses to perform critical computational functions in neural circuits. It is clear that we cannot understand neural coding or information processing without taking synaptic dynamics into account. Here, we review some of the forms of synaptic plasticity and discuss their implications for neuronal coding and signalling.

Expression and induction of plasticity

Synapses transmit information when presynaptic action potentials cause the membrane fusion of neurotransmitter-containing vesicles. This is followed by binding of the released transmitter to receptors that modify postsynaptic activity^{8–10}. On rapid timescales (milliseconds to minutes) the release of neurotransmitter depends on the pattern of presynaptic activity, and synapses can be thought of as filters with distinctive properties. This provides synapses with computational potential and has important implications for the diversity of signalling within neural circuits. Neural responses are typically described by specifying the sequences of action potentials that neurons fire. Such sequences are used to characterize the selectivities and information content of neuronal responses, and they form the basis of virtually all studies of neural coding. Implicit in this approach is the assumption that individual neurons ‘speak with a single voice’. This ‘voice’ consists of the action potential sequences that would, for example, be recorded from the neurons in standard electrophysiology experiments. The remarkable range and variety of synaptic plasticity mechanisms make this single voice, ‘spikes equal signal’ assumption untenable.

Synapses from the same neuron can express widely different forms of plasticity^{11–13}. Moreover, connections between neurons can sometimes consist of a single release site^{12,14} where the release of neurotransmitter is probabilistic

and the likelihood of release is modified by activity through short-term plasticity. Such synapses selectively, although unreliably, filter the flow of information between neurons. Given the stochastic nature of transmission, a neuron firing a sequence of action potentials is likely to generate a different pattern of vesicle releases at each of its thousands of synaptic terminals. So, each neuron transmits not just one, but a large number of different signals to the neural circuit in which it operates. Individually, these synapse-specific signals are selectively filtered versions of the action potential sequence that the neuron generates, modified by the context of previous presynaptic and postsynaptic activity. Collectively, knowing which synapses transmit a given action potential — the signal by which neurons interact — provides more information than simply knowing that a neuron has fired. Communication from a single neuron is thus a chorus not a single voice.

Just as the expression of synaptic plasticity involves a huge range of timescales, its induction can be rapid or can involve integration of activity over long periods of time. Induction requirements for synaptic plasticity can impose complex contingencies on the temporal patterns of activity that maximize effective circuit connectivity. The potential computational power of synapses is large because their basic signal transmission properties can be affected by the history of presynaptic and postsynaptic firing in so many different ways^{7,15}. Three classes of induction requirements can be identified depending on the direction of information flow across the synapse. The basic process of synaptic transmission is feedforward, with the presynaptic neuron sending a signal to its postsynaptic target (downward in Fig. 1a, b). Several forms of plasticity are feedforward in character, meaning that their induction depends solely on presynaptic activity (right-pointing arrows in Fig. 1b). Such forms of plasticity are the main focus of this review. However, the flow of information across a synapse can also be bidirectional, which greatly enhances computational potential. Synaptic plasticity can depend on feedback from the postsynaptic neuron (upward in Fig. 1b) through the release of retrograde messengers^{16,17} (left-pointing arrows in Fig. 1b). This ‘feedback plasticity’ may operate in isolation or in conjunction with presynaptic activity (associative plasticity). Feedforward, feedback and associated forms of synaptic plasticity have quite different functional and computational implications.

Forms of synaptic plasticity

Many factors affect how a postsynaptic neuron responds to the arrival of a presynaptic action potential at a particular synapse. On the postsynaptic side, receptor desensitization, in which prolonged exposure to the neurotransmitter inactivates

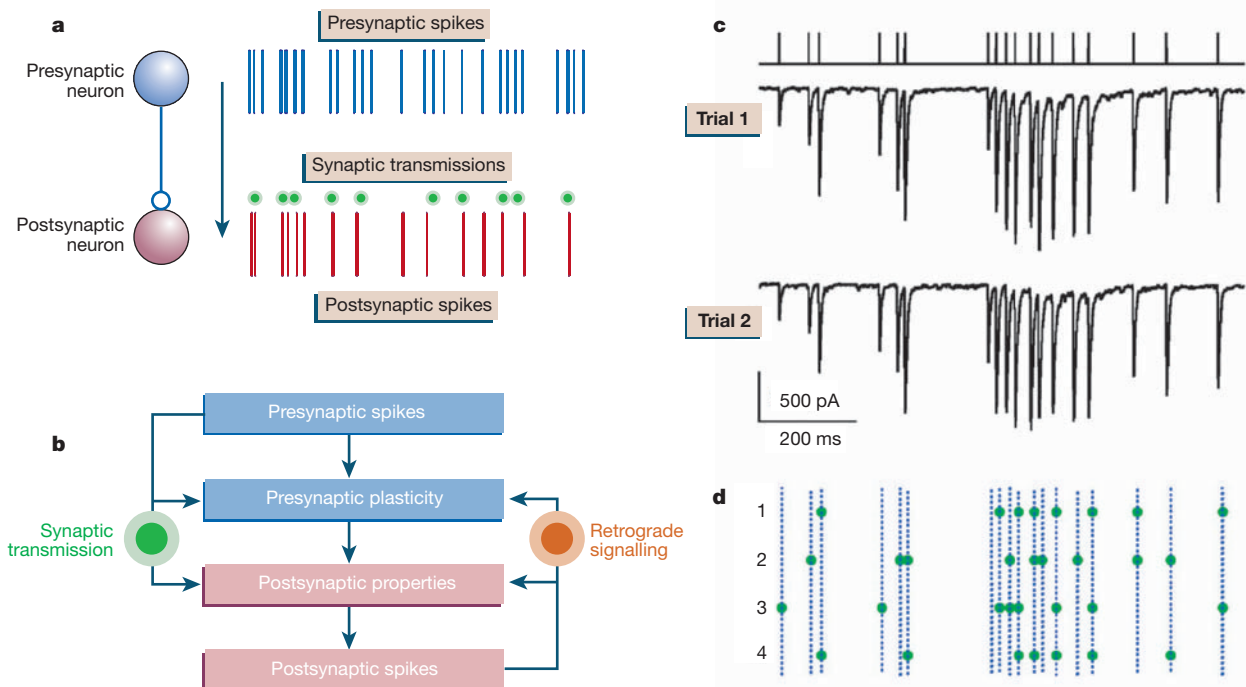


Figure 1 Several processes determine how a presynaptic neuron influences the firing pattern of its postsynaptic targets. **a**, Representative firing patterns of presynaptic and postsynaptic neurons. Blue lines denote presynaptic spikes; green dots denote synaptic vesicle releases (that is, transmissions); and red lines denote postsynaptic spikes. **b**, Pathways through which the firing patterns of presynaptic and postsynaptic neurons influence each other and synaptic transmission, including feedforward (pre-to-post) and feedback (post-to-pre) effects. **c**, **d**, Influence of short-term synaptic plasticity on synaptic transmission evoked by irregular stimulus

trains. **c**, Stimulation from the same stimulus train over two trials results in similar synaptic currents (top). These synaptic currents are measured in cerebellar Purkinje cells in response to parallel fibre activation. **d**, Simulated vesicle releases at an individual bouton over four trials with the same stimulus train as in **c**. Stimulus timing is indicated by the vertical blue lines and vesicle release is indicated by a green dot. Time bar is the same as in **c**. The occurrences of vesicle fusions were not measured in these experiments but are included to illustrate what probably occurs at individual release sites. Fig. 1c adapted from ref. 48.

receptors, decreases the ability of the postsynaptic cell to respond to the neurotransmitter^{18–22}. The type of receptor activated at the synapse also affects the postsynaptic response. Glutamate, for example, can activate AMPA receptors, NMDA receptors, and metabotropic glutamate receptors (mGluRs)¹⁰. AMPA receptors show a range of properties but usually have rapid kinetics. NMDA receptors have much slower kinetics and are voltage dependent. mGluRs are coupled to second messenger systems that can lead to modulation and activation of channels and to the release of calcium from internal stores²³. Finally, the location of a synapse on the dendritic arbor in relation to the general morphology of the neuron and its distribution of active conductances, as well as the presence of other active synapses, all have important roles in determining the postsynaptic response^{24,25}.

We cannot cover all the factors that contribute to the transformation from a presynaptic action potential to a postsynaptic response in this review. Because we are interested in the computational potential of dynamic synapses, we will focus on plasticity at the synapse: activity-dependent changes in the probability of vesicle release and in the response of postsynaptic receptors. Numerous mechanisms of plasticity acting over a wide range of timescales influence the release of neurotransmitter-containing vesicles. The initial probability of release and use-dependent plasticity of synapses are determined by the identities of the presynaptic and postsynaptic neurons, as well as by the history of action potential activity and by the local environment^{26,27}. There are numerous examples of boutons from the same axon giving rise to facilitating synapses (that enhance synaptic strength) for some types of target neurons and to depressing synapses (that reduce synaptic strength) at others^{13,27}. The target can also induce the expression of distinctive modulatory receptors in presynaptic boutons²⁶. These findings indicate that the postsynaptic cell

influences the presynaptic properties of the synapse, either through direct contact or by liberating a retrograde messenger. There is, however, considerable diversity in the properties of synaptic connections between two cell types, indicating that additional refinement of synaptic properties can occur. The dynamic properties of synapses are also refined in a use-dependent manner by long-term mechanisms of synaptic plasticity.

Feedforward plasticity

Periods of elevated presynaptic activity can cause either an increase or a decrease in neurotransmitter release⁷. Facilitation reflects an increase in the probability of neurotransmitter release (p) that lasts for up to hundreds of milliseconds. Depression reflects a decrease in the probability of neurotransmitter release that persists for hundreds of milliseconds to seconds. Facilitation and depression seem to coexist at synapses, with their relative weight depending largely on the initial p : high p favours depression, low p favours facilitation. On longer timescales (tens of seconds to minutes), longer-lasting forms of depression reduce synaptic strength and augmentation and post-tetanic potentiation (PTP) enhance it. Repeated presynaptic activation is typically required to produce appreciable synaptic plasticity. Several forms of these longer-lasting types of enhancement and depression coexist at most synapses.

To understand how short-term plasticity affects how a presynaptic neuron influences the firing of its postsynaptic targets (Fig. 1a), it is useful to activate synaptic inputs and record the responses in whole-cell voltage clamp. In Fig. 1c, synaptic inputs are activated with an irregular stimulus train of the sort that might occur at many types of synapse *in vivo*. Synaptic currents start out small, increase in amplitude during high-frequency bursts and then decrease following

quiescent periods. In the two trials shown in Fig. 1c, the responses are remarkably stereotyped and there is relatively little variability. This is because the response is mediated by many tens of synaptic contacts. If, on the other hand, the response of individual synaptic contacts is considered, stochastic variability becomes important (Fig. 1d). Release patterns from individual boutons in response to the same pattern of stimulation vary considerably, as is illustrated by the four simulated traces in Fig. 1d. Similarly, on single trials the same stimulus can evoke different patterns of transmitter release at different synaptic contacts. But despite large trial-to-trial variations, the facilitation present at this synapse can be seen in Fig. 1d. Greater enhancement of release can be seen during high-frequency bursts than following periods of inactivity.

Feedback plasticity

Recent studies have also identified plasticity operating on rapid timescales that depends on postsynaptic activity^{28–31}. Several retrograde messengers have been identified that once released from dendrites act on presynaptic terminals to regulate the release of neurotransmitter^{16,31–34}. The endocannabinoid system is the most widespread signalling system that mediates retrograde signalling¹⁶. Endocannabinoids are released from the postsynaptic cell following the cleavage of lipid precursors. This release of endocannabinoids leads to an inhibition of neurotransmitter release that lasts for tens of seconds^{35–37}. Endocannabinoid release can be triggered by increased concentrations of calcium in postsynaptic cells and by activation of second messengers systems. This suggests that the state of the postsynaptic cell exerts control on neurotransmitter release from the presynaptic terminals by regulating the release of endocannabinoids.

The roles of retrograde inhibition by endocannabinoids are not yet well understood. One possibility is that this inhibition provides a general means for postsynaptic neurons to control the inputs they receive, providing homeostatic regulation of synaptic strength based

on postsynaptic activity levels. Although an intriguing possibility with parallels on longer timescales, the exceptionally high calcium concentrations required for calcium-dependent endocannabinoid release³⁸ make it unlikely that endocannabinoids normally operate in this manner. Instead it seems that endocannabinoids can lead to synapse-specific modulation³⁹. For example, burst firing in presynaptic cells can evoke local endocannabinoid release and selective synaptic regulation. One interesting, yet to be tested, possibility is that endocannabinoids provide a mechanism of short-term associative plasticity (as is the case for long-term plasticity^{40–42}), in which endocannabinoid release and synaptic modulation are controlled by postsynaptic and presynaptic activity.

Associative plasticity

Short-term forms of associative plasticity would be useful for several reasons⁴³. Network models based on short-term plasticity can lead to persistent activity in a subset of neurons that represent a particular memory. Models based on fast associative plasticity are more robust than models relying solely on finely tuned synaptic weights within the network⁴⁴. Rapid associative plasticity could also be useful for improving performance on a task where predictions are made and then error signals are used to correct deviations from those predictions⁴⁵. This is because associative plasticity allows the error signal to make appropriate corrections by modifying synapses that lead to incorrect performance. Despite these potential uses of short-term associative plasticity, in contrast to the many associative forms of long-term depression and potentiation (LTD and LTP) that have been identified, far less is known about synaptic mechanisms that could implement associative plasticity on the seconds to tens of seconds timescale.

Functional roles of short-term plasticity

A number of functional roles have been proposed for synaptic dynamics^{46–61}. Short-term synaptic plasticity can drastically alter how a neuron activates its postsynaptic targets^{48,62}. Figure 2 compares the variety of ways that different synapses respond to patterns of spiking. In these examples, the synaptic responses are measured in voltage-clamp mode and the postsynaptic cell is not allowed to fire an action potential, although it is clear that synapses with such different dynamics would lead to very different postsynaptic firing. The climbing fibre synapse has a high initial p and therefore depression dominates the short-term plasticity during bursts, with gaps in the presynaptic activity allowing recovery. Parallel fibre synapses are low p synapses and facilitation dominates their short-term plasticity, with relaxation occurring during pauses in presynaptic activity. Hippocampal Schaffer collateral synapses have an intermediate p and show a large transient enhancement of synaptic strength but a less pronounced steady-state level of enhancement.

Patterns of activation and details of spike timing have a profound influence on synaptic strength. For these synapses, the interplay between multiple forms of plasticity determines the response properties of the synapses. This interplay exists when either depression or facilitation is dominant, but it is most apparent when the initial probability of release is intermediate (when both depression and facilitation are prominent). In all cases, the timing of synaptic activation matters and the use dependence is important in conveying information about the timing and structure of the presynaptic train to the postsynaptic cell.

Synaptic filtering

An important consequence of synaptic dynamics is that synapses can act as filters with a wide range of properties^{48,50,51,57}. This is readily appreciated by plotting steady-state responses as a function of stimulus frequency (Fig. 2b). Synapses with a low initial probability of neurotransmitter release, such as parallel fibre synapses, function as high-pass filters, whereas synapses with a high initial probability of release, such as climbing fibre synapses, act as low-pass filters that are

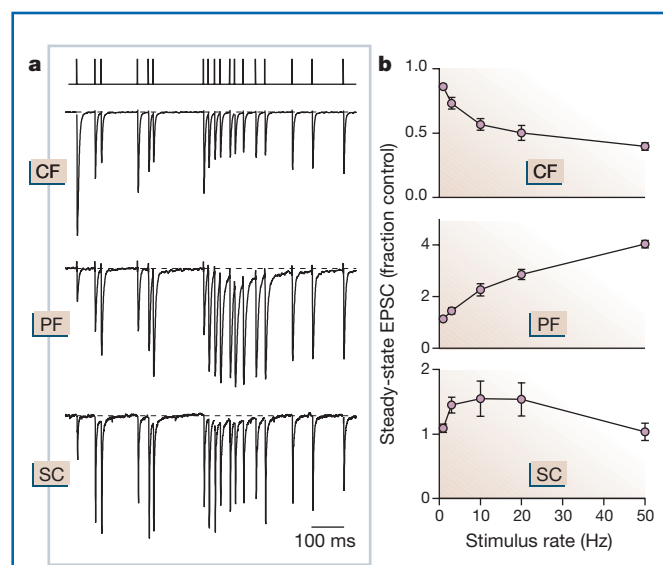


Figure 2 Examples of excitatory postsynaptic currents (EPSCs) recorded in response to an irregular stimulus train with an average rate of 20 Hz at the climbing fibre, parallel fibre and Schaffer collateral synapses. These results illustrate low-pass (climbing fibre), high-pass (parallel fibre) and band-pass (Schaffer collateral) filtering characteristics. **a**, Diversity of short-term plasticity. Top, climbing fibre to Purkinje cell EPSCs; middle, parallel fibre to Purkinje cell EPSCs; bottom, CA3 to CA1 Schaffer collateral EPSCs. Traces are averages of four to six trials. **b**, Average magnitude of the eighth to tenth EPSC from a regular train, normalized by the first EPSC and plotted as a function of stimulus frequency for climbing fibre (top), parallel fibre (middle) and Schaffer collateral (bottom) synapses. Adapted from ref. 48.

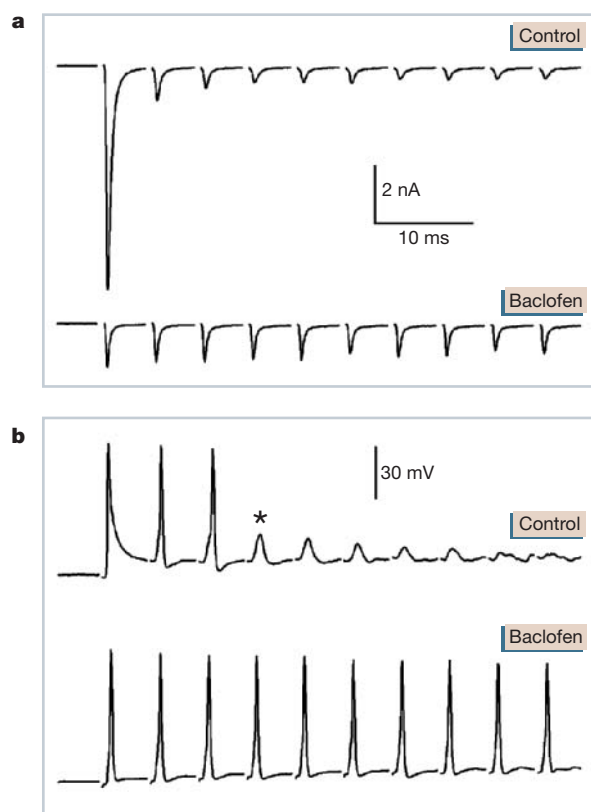


Figure 3 Synaptic modulation regulates synaptic dynamics and influences the transmission function of synapses. Presynaptic modulation with the GABA (γ -aminobutyric acid) receptor agonist baclofen affects the response of the end-bulb synapse formed by auditory nerve terminals onto the soma of neurons in the avian nucleus magnocellularis. Responses to high-frequency stimulus trains were measured by voltage clamp (**a**) and by current clamp (**b**). In **a**, although the synaptic current evoked by the initial stimulus is greatly inhibited by baclofen, responses late in the train are larger in baclofen-treated synapses than in control synapses. **b**, In current clamp stimulation during high-frequency trains, transmission is more reliable in baclofen-treated synapses than in control synapses. In control conditions, only the first two stimulus pulses trigger spikes and subsequent spikes fail, whereas in baclofen-treated synapses there are only three spike failures in the entire train. (Asterisk indicates examples of failures to produce spikes.) Adapted from refs 63, 65.

most effective at the onset of presynaptic activity. Synapses with an intermediate probability of release, such as Schaffer collateral synapses, act as band-pass filters that are most effective at transmitting impulses when there is an intermediate range of presynaptic activity.

The filtering characteristics of a given synapse are not fixed; they can be adjusted through modulation of the initial release probability or other aspects of synaptic transmission⁴⁸. Many neuromodulators activate presynaptic receptors, and the result is often a reduction in the probability of release. As a result of this decrease in the amount of neurotransmitter released, the filtering characteristics of the modulated synapse are altered so that depression makes a smaller contribution to synaptic dynamics and facilitation becomes more prominent. In this way, presynaptic inhibition can convert a synapse from a low-pass filter to a band-pass filter, or from a band-pass filter to a high-pass filter.

In some circumstances, the interaction of different forms of synaptic plasticity can cause modulation to have counterintuitive effects. For example, at the end-bulb synapse formed by auditory nerve terminals onto the soma of neurons in the avian nucleus magnocellularis, presynaptic inhibition greatly reduces the initial synaptic current evoked during a train, but for high-frequency activation there is less steady-state reduction of release than would be expected

(Fig. 3a). In this case, presynaptic inhibition paradoxically results in a synapse that is more effective at inducing the postsynaptic cell to fire spikes during a high-frequency train⁶³ (Fig. 3b). This behaviour arises because this synapse is particularly prone to receptor desensitization when the probability of release is high^{64,65}. By reducing the probability of release, presynaptic inhibition causes less desensitization and therefore the 'inhibition' actually increases the effective strength of the synapse during ongoing high-frequency activation.

Adaptation and enhancement of transients

Neurons typically respond most vigorously to new rather than to static stimuli. Synaptic depression provides a possible explanation for this virtually universal feature of sensory processing. Consider the case of sensory input to a neuron A that in turn excites neuron B through a depressing synapse. Even if a prolonged sensory stimulus activates neuron A in a sustained manner, the response of neuron B may only be prominent at the onset of stimulation because synaptic depression produces a synapse-specific decrease in the drive to neuron B. This results in a neuron that only responds to new stimuli. Synaptic depression acting in this manner may contribute to contrast adaptation⁶⁶ and to suppression by masking stimuli^{67,68} in primary visual cortex.

Decorrelation and burst detection

Figure 4 shows a sample presynaptic spike train along with the pattern of transmission it evokes from two types of model synapse. Both model synapses have time-dependent transmission probabilities, but one shows depression and the other facilitation (see Fig. 4 legend for details). Both transmit about 25% of the presynaptic action potentials at the average presynaptic firing rate shown (35 Hz), but their pattern of transmission differs. The depressing synapse produces transmission sequences that are more regular than those generated by the facilitating synapse. The coefficient of variation for the inter-transmission intervals of the facilitating synapse is more than double that for the depressing synapse (1.5 for facilitating synapse; 0.7 for depressing synapse). The transmissions produced by

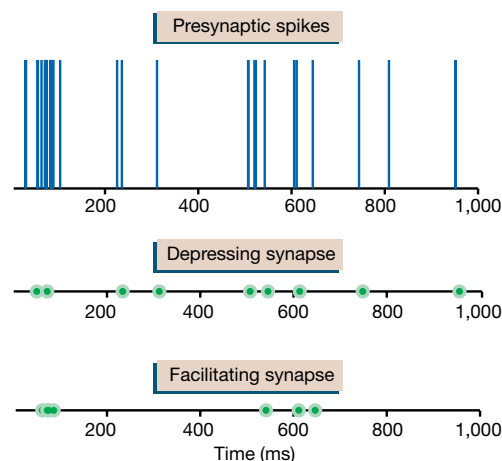


Figure 4 Stochastic transmission from two model synapses. A presynaptic spike train (blue lines) induces the sequences of transmissions in these two synapses (green dots). One model synapse shows depression and the other facilitation. Immediately after a successful transmission, the transmission probability for the depressing synapses is set to zero. The probability then recovers exponentially back towards one with a time constant of 200 ms. The facilitating synapse has a resting transmission probability of zero in the absence of presynaptic activity. Each presynaptic action potential reduces the distance between the value of the transmission probability and its maximum allowed value of one by 10%. Between presynaptic spikes, the transmission probability for the facilitating synapse decays exponentially back towards zero with a time constant of 50 ms.

depressing synapses tend to be more regular and less positively correlated than the presynaptic spike sequences that evoke them. Because of this, synaptic depression has been proposed as a mechanism that removes redundant correlations so that transmission sequences convey information in a more efficient manner⁵⁹. Facilitating synapses tend to produce transmission sequences that are more irregular and more positively correlated than the presynaptic spike trains that evoked them because facilitation favours burst-like clusters of transmissions. This suggests that facilitation could enhance information coding that is mediated by bursts of action potentials⁶⁰.

Information flow

Transmission across a synapse is obviously the conveyance of information carried in a presynaptic action potential to the postsynaptic neuron. However, for dynamic synapses each synaptic transmission also contains information about the previous history of spiking. This contextual information can be quantified^{49,69,70}. Synaptic plasticity assures that current activity reflects both the current state of a stimulus and the previous history of activity within the neural circuit. Neuronal adaptation can also contribute to this effect, but synaptic plasticity has the advantage of carrying information which is specific to the activity of an individual presynaptic neuron.

Sound localization

Synaptic depression may also have an important role in sound localization^{71,72}. In the avian brain, neurons in nucleus laminaris (NL) represent the spatial location of a sound. Firing of NL neurons requires precisely coincidental arrival of binaural input, and results in high sensitivity to differences in sound conduction delays between the

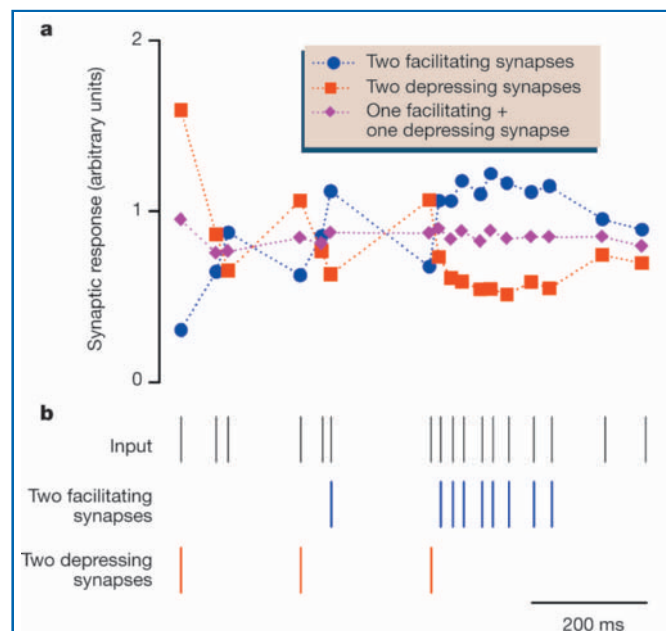


Figure 5 The ability of coactivated synapses to activate their targets depends on whether the synaptic inputs have the same use-dependent plasticity. **a**, The amplitudes of synaptic currents resulting from the simultaneous stimulation of two inputs: both facilitating, both depressing or one of each type. If the synapses share the same type of plasticity they reinforce each other's variations in synaptic strength. In contrast, if a facilitating and a depressing synapse are activated, their variations in synaptic strength tend to cancel each other out. This affects the ability of the synapses to fire their targets. **b**, The amplitudes of the responses cross a threshold and fire the postsynaptic cell during high-frequency bursts of two facilitating synapses (blue) and following pauses in presynaptic activity for two depressing synapses (red). The combination of a facilitating and a depressing synapse gives a relatively uniform input that is unable to evoke any postsynaptic spikes (not shown).

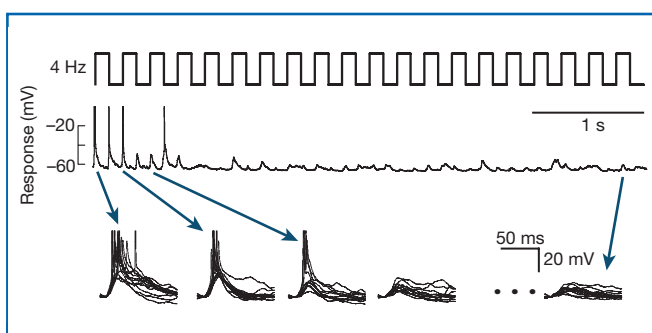


Figure 6 Synaptic depression of thalamocortical synapses underlies sensory adaptation in the cortex. The primary whisker of a rat is stimulated at 4 Hz (top) and the response of a cortical neuron in the corresponding region of barrel cortex is measured with an intracellular recording electrode (middle). Even though whisker stimulation is maintained, action potentials are only evoked in the cortical cell during the first second of stimulation. This stimulation is repeated 12 times. An expanded view of the responses observed in the cortical cell during different periods of stimulation (bottom) shows that as the train progresses, EPSPs became progressively smaller and eventually are no longer able to evoke action potentials. Extensive experiments suggest that synaptic depression at the thalamocortical synapse underlies the sensory adaptation observed during whisker stimulation. Adapted from ref. 75.

two ears, and so to sound location⁷³. These neurons localize sounds over a broad range of intensities. Increases in sound level elevate the firing rates of the inputs to NL neurons, suggesting that intensity could be a complicating factor in spatial discrimination. Synaptic depression of the inputs onto NL neurons provides a possible explanation for how sound localization operates over a broad range of intensities^{71,72}. Although a louder sound provides higher frequency inputs to NL neurons, this effect is offset by synaptic depression. As a result, the total synaptic input delivered is independent of stimulus frequency and therefore independent of sound intensity.

Dynamic input compression

Neurons integrate thousands of inputs, each firing over a range of about 1–100 Hz. But they keep their output firing rates within this same range. Doing this requires precise mechanisms of gain control and input compression. Sensory systems face similar compression problems owing to the enormous range of intensities found in nature for most stimuli. Many sensory responses obey a Weber–Fechner law, meaning that changes in stimulus intensity are interpreted in relative or percentage terms rather than on an absolute scale. This results in a logarithmic compression of the intensity scale. Synaptic depression seems to allow a similar form of compression to occur at the neuronal level^{52,74}. This is because, when depression is occurring, the level of synaptic transmission at high rates is proportional to the inverse of the presynaptic firing rate. A rapid change in the presynaptic firing rate thus results in a transient synaptic current that is proportional to the size of that change scaled by the baseline firing rate.

Interactions of synaptic inputs

Neural responses typically arise from the summation and interaction of several synaptic inputs. Figure 5 shows the response of a neuron to two synaptic inputs with various forms of short-term plasticity. Two depressing synapses produce the largest synaptic responses after long periods of presynaptic inactivity (Fig. 5a; red squares), whereas two facilitating synapses are most effective at transmitting at the end of a burst of activity (Fig. 5a; blue circles). In contrast, the plasticity of a depressing synapse counteracts the plasticity of a facilitating synapse, so the summed output of a facilitating and a depressing synapse shows fewer pronounced use-dependent alterations in amplitude (Fig. 5a; purple diamonds).

The degree to which coactivated synapses share properties of short-term plasticity influences their ability to stimulate postsynaptic

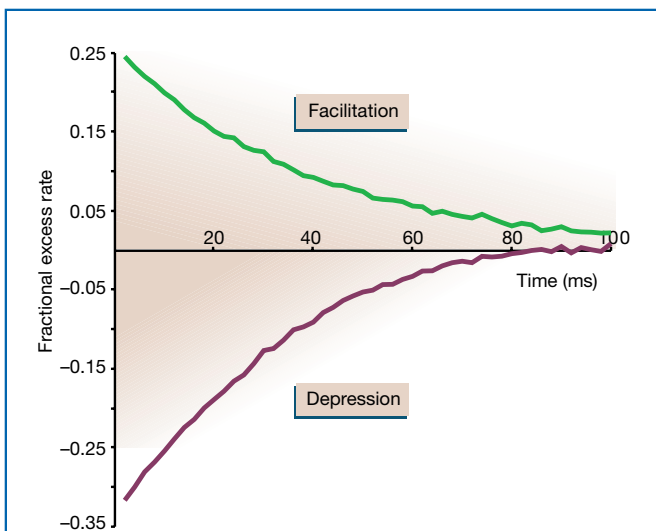


Figure 7 The fractional excess in presynaptic firing rate at different times before a transmission at the facilitating (green curve) and depressing (red curve) model synapses of Fig. 4.

targets (Fig. 5b). In Fig. 5, if the two inputs both facilitate, they trigger the postsynaptic cell to fire late in a burst (Fig. 5b; blue bars); if the two inputs both depress, they trigger spikes following periods of inactivity in the presynaptic cells (Fig. 5b; red bars). Finally, if one facilitates and the other depresses, no spikes at all are triggered in the postsynaptic cell (not shown). These results indicate that two or more cells that fire with a given pattern of activity are more effective at influencing their postsynaptic targets if they exhibit the same type of synaptic plasticity, owing to mutual reinforcement.

Synaptic depression *in vivo*

Experimental studies of synaptic properties in brain slices and theoretical considerations have established numerous potential roles for synaptic plasticity. Establishing the function of such short-term plasticity *in vivo* has been more difficult, but a recent study showed that this is possible⁷⁵. Neurons in somatosensory cortex respond to initial whisker stimulation but they stop responding to repeated stimulation (Fig. 6). Such sensory adaptation is useful in that only novel stimuli are able to evoke robust responses and repeated stimuli can be ignored. *In vivo* whole-cell recordings established that depression at thalamocortical synapses was responsible for this sensory adaptation⁷⁵. Repeated whisker stimulation led to repeated synaptic activation, and depressed synaptic responses to such an extent that they were no longer able to activate cortical neurons.

Characterizing synaptic filtering

The stochastic filtering of the presynaptic spike train that dynamic synapses perform can be characterized by computing the average pattern of presynaptic spiking that precedes a synaptic transmission. In other words, we can count the number of presynaptic action potentials that occur within specified time intervals (bins) centred at various times before each synaptic transmission over a long period of spiking, and divide this by the number of transmissions and the width of the time bins being used. The result gives the average temporal evolution of the firing rate of the presynaptic neuron before a synaptic transmission. Next, we subtract the time-averaged firing rate of the presynaptic neuron from this time-dependent firing rate, and again divide the result by the number of transmissions and the width of time bins used. This produces the plots of average fractional excess presynaptic firing rate before a synaptic transmission shown in Fig. 7. The bin at zero is omitted from this plot because it is very large and positive. This reflects the fact that there is always a presynaptic action

potential at the time of a synaptic transmission; it is the action potential that evokes the transmission. If the synapse had no intrinsic dynamics, the excess presynaptic firing rate would be zero for all the other bins plotted.

Not surprisingly, facilitating synapses typically transmit after periods of excessive presynaptic spiking (Fig. 7, green line), and depressing synapses transmit preferentially after periods of less-than-average spiking (Fig. 7, red line). Interestingly, the curve for the facilitating synapse decays to zero more slowly than the curve for the depressing synapse even though the recovery time for depression is considerably greater than that for facilitation in these models (200 ms versus 50 ms). This is because facilitation builds up on each pre-synaptic spike, whereas depression occurs only when there is a successful transmission.

If we keep in mind that there is a sharp upward spike in the curves of Fig. 7 at the zero time point (which has been omitted for clarity), it is apparent that the depressing synapse performs an approximation of differentiation, and that the facilitating synapse performs a short-term integration. A linear filter that approximates differentiation would have a sharp positive spike at time zero and a matching sharp negative spike after a short time. The negative portion of the red curve in Fig. 7 is not a sharp spike, which means that differentiation by the depressing synapse is a low-pass filtered approximation. The integration being performed by the facilitating synapse is of a similarly leaky form.

The transmission-triggered average

The curves in Fig. 7 characterize the selectivity of depressing and facilitating synapses in terms of presynaptic spike sequences. Neuronal selectivity, however, is typically characterized in terms of the stimulus used to evoke a response. One of the most powerful and widely used methods for characterizing such neural selectivity is the 'spike-

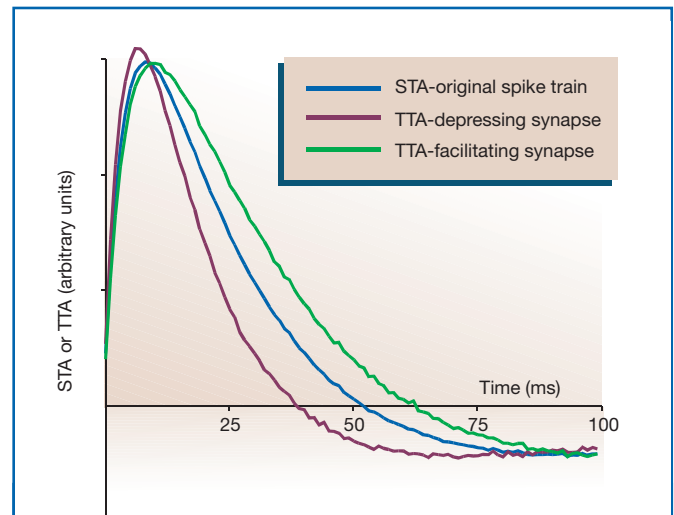


Figure 8 STAs and TTAs for a model neuron. The blue curve is an STA of a white-noise stimulus plotted against time before the triggering action potential. The red and green curves are TTAs obtained in the same way as the STA, but with the stimulus averaging triggered on each transmission from either a depressing (red curve) or a facilitating (green curve) synapse. For all three traces, a model neuron is driven by a white-noise stimulus to produce a sequence of action potentials. The model neuron consists of a linear filter, chosen to match typical temporal response properties of sensory neurons, providing input to a Poisson spike generator. To obtain the STA, the white-noise stimulus is sampled before each action potential is generated by the model neuron, and these samples are averaged over a long period of spiking. To compute TTAs, the spike sequence generated by this model neuron is fed into the model synapses shown in Fig. 4. Each time a presynaptic spike results in a transmission, the preceding stimulus is sampled, and an average taken over all transmissions.

triggered average' (STA). In this procedure, a stimulus is used (usually of the white-noise variety) to activate a neuron and the evoked action potentials are recorded. The STA stimulus is then computed by sampling the stimulus for a period of time before each action potential and then by averaging the samples obtained in this manner over all the recorded action potentials. The STA thus characterizes what 'typically' happens before a spike, and it is a standard measure of neuronal selectivity.

An extension of the concept of the STA that is useful for our discussion of synaptic dynamics is the 'transmission-triggered average' (TTA). To compute a TTA we compute the average stimulus that occurs before each transmission at a given synapse. By doing this for individual synapses showing different forms of plasticity, such as depression or facilitation, we can explore forms of selectivity that are relevant to neural circuits but that cannot be detected directly by conventional methods of experimental electrophysiology.

Figure 8 provides a comparison of a conventional STA with TTAs for two types of model synapses. The STA (Fig. 8, blue curve) shows that the model neuron is particularly responsive to positive values of the stimulus that occur about 5–30 ms before an action potential. For even earlier times relative to the action potential (50–100 ms before the action potential), the neuron responds preferentially to negative stimuli. Such reversals of selectivity over time are often seen in the temporal receptive fields of sensory neurons. For more than 150 ms before an action potential the STA goes to zero (not shown). The TTAs for the depressing and facilitating synapses are indicated by the red and green curves in Fig. 8. The red curve, corresponding to the depressing synapse, shows sensitivity to the stimulus over a shorter time period than the STA would imply, whereas the green curve, corresponding to the facilitating synapse, reveals a longer lasting sensitivity to the stimulus. Note that the temporal selectivity that we would normally infer for this neuron, given by the STA (blue curve), does not correctly characterize the selectivity seen by postsynaptic targets connected by synapses displaying these types of plasticity. Postsynaptic targets connected through depressing synapses receive a signal corresponding to a short temporal integration of the stimulus, whereas other targets connected by facilitating synapses receive a signal corresponding to a longer temporal integration period.

The key point is that the temporal selectivity for a neuron that transmits through synapses showing different forms of plasticity cannot be characterized by a single temporal receptive field function. Normally the STA (Fig. 8, blue curve) would be used for this purpose but, depending on the particular target being considered, either the red or the green TTA provides a more accurate measure of the temporal selectivity of this neuron. Characterizing the total signal that this neuron delivers to its postsynaptic targets would require an entire family of TTAs with a variety of forms.

Conclusion

The firing pattern of a group of neurons is often used to describe the 'state' of a neural circuit, but this description is clearly incomplete. To predict how a circuit will respond to a stimulus and to interpret that response, we also need to know the dynamic state of its synapses. Given that there are many more synapses than neurons in a typical circuit, the state of a neural network might better be described by specifying the state of its synapses than the firing pattern of its neurons. We might even extend this viewpoint by stating that the role of synapses is to control neuronal firing within a neural circuit, but the role of neural firing is to set the states of the synapses. Experimental and theoretical approaches to the problem of synaptic computation are beginning to put neurons and synapses on a more equal footing with regard to their roles in neural computation. □

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Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death

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Huntington's disease is caused by an abnormal polyglutamine expansion within the protein huntingtin and is characterized by microscopic inclusion bodies of aggregated huntingtin and by the death of selected types of neuron. Whether inclusion bodies are pathogenic, incidental or a beneficial coping response is controversial. To resolve this issue we have developed an automated microscope that returns to precisely the same neuron after arbitrary intervals, even after cells have been removed from the microscope stage. Here we show, by survival analysis, that neurons die in a time-independent fashion but one that is dependent on mutant huntingtin dose and polyglutamine expansion; many neurons die without forming an inclusion body. Rather, the amount of diffuse intracellular huntingtin predicts whether and when inclusion body formation or death will occur. Surprisingly, inclusion body formation predicts improved survival and leads to decreased levels of mutant huntingtin elsewhere in a neuron. Thus, inclusion body formation can function as a coping response to toxic mutant huntingtin.

Huntington's disease (HD), a neurodegenerative disorder caused by an abnormal polyglutamine (polyQ) expansion within the protein huntingtin (Htt), is characterized by the aggregation of Htt into microscopic intracellular deposits called inclusion bodies (IBs) and by the death of striatal and cortical neurons. However, the relationship between Htt deposition and neurodegeneration is controversial. Sometimes IB formation has been associated with neurodegeneration^{1–4}; at other times, there was no or a negative correlation^{5–9}. Three competing models have described IB formation as pathogenic, incidental or a beneficial coping response^{5,10–12}.

IBs seem to result from aggregation that generates many protein complexes differing in multimerization and three-dimensional structure¹³. These complexes often coexist with IBs, but low temporal and spatial resolution have limited the interpretation of past experiments that correlated IB formation with neurodegeneration. Attempts to disrupt the aggregation process yielded opposing results, depending on the manipulation^{5,7,9,14–16}, probably because it is impossible to manipulate aggregation selectively¹⁷.

Automated microscopy of a model for HD

To increase the temporal resolution of conventional approaches, we developed an automated microscope system¹⁸ that returns to precisely the same neuron or field of neurons, even after cells have been removed from the microscope stage during the interval. We prospectively measured the survival of individual neurons, the intracellular levels of mutant Htt and the aggregation of Htt into IBs. The relationships between these factors were determined by survival analysis without introducing potentially confounding nonspecific manipulations^{19,20}.

We examined an established neuronal HD model⁵ in which striatal neurons are transiently transfected with Htt. The model recapitulates several HD features (for example, IB formation and polyQ-expansion-dependent, neuron-specific death)⁵. To reveal Htt in living striatal neurons, we used amino-terminal exon 1 fragments of Htt (Htt^{ex1}) containing polyQ stretches of various lengths and

fused to the N terminus of green fluorescent protein (GFP)²¹. A similar fragment may be generated in HD by proteolytic cleavage^{22–26} and is sufficient to produce HD-like features when expressed as a transgene in a mouse²⁷. Along with Htt^{ex1}-GFP, neurons were co-transfected with a monomeric red fluorescent protein (mRFP)²⁸ to reveal neurons independently of Htt^{ex1}-GFP (Supplementary Fig. S1). Fluorescent protein expression and periodic imaging did not affect neuronal viability¹⁸.

Neurons were imaged with the automated microscope 2–24 h after transfection and at 12–24-h intervals (Fig. 1a). Some neurons abruptly lost mRFP fluorescence. This event corresponded to the loss of membrane integrity and cell death and was well correlated with other cell-death markers (Fig. 1b, Supplementary Fig. S2). Others have found the loss of a fluorescent marker protein to be a highly sensitive and specific assay of cell death through different pathways and in different types of cell²⁹. The ability to monitor individual neurons over time allows us to quantify differences in their longevity by survival analysis^{19,20}. We determined the survival function for neurons transfected with GFP or with Htt^{ex1}-GFP containing a normal (Q₁₇) or expanded (Q₇₂) polyQ stretch. Neurons transfected with Htt^{ex1}-GFP containing disease-associated polyQ stretches died faster than neurons transfected with Htt^{ex1}-Q₁₇-GFP (Fig. 1c).

From the survival functions, we deduced hazard functions—the estimated instantaneous risk of death of individual cells, independent of population size^{19,20}. The cumulative risk of death was similar and remained relatively low in neurons transfected with GFP or Htt^{ex1}-Q₁₇-GFP (Fig. 1d, Supplementary Fig. S3). However, Htt^{ex1}-Q₄₇-GFP, Htt^{ex1}-Q₇₂-GFP or Htt^{ex1}-Q₁₀₃-GFP significantly increased the risk, and the increase was correlated with the length of the polyQ stretch. These results parallel features of HD: polyQ stretches longer than 35Q can cause neurodegeneration, with symptoms appearing sooner for longer stretches³⁰.

Knowing whether the risk of death changes over time can provide insights into the mechanisms responsible for neurodegeneration³¹. The cumulative risk of death increases as cells continually die

(Fig. 1), but the risk of cell death does not necessarily change. To determine whether the risk of death changes, we tested the linearity of the non-cumulative hazard function: a curved function means that the risk of death changes over time; linearity indicates that the risk is largely time-independent. The hazard functions for neurons transfected with Htt^{ex1}-Q₄₇-GFP, Htt^{ex1}-Q₇₂-GFP or Htt^{ex1}-Q₁₀₃-GFP were essentially linear (*F*-test, not significant), indicating that the expanded polyQ stretches increase the risk of death relatively constantly over time.

However, these cultures contain subtypes of striatal neuron whose susceptibility varies in HD^{5,32,33} and could mask a temporal variation in the risk of death conferred by polyQ expansion. We therefore performed parallel experiments in a homogeneous, neuron-like pheochromocytoma 12 (PC12) cell line (Fig. 1e, f). PC12 cells containing versions of Htt with disease-associated polyQ expansions had a higher risk of death than those containing versions of Htt with wild-type polyQ expansions; the increase was relatively constant over time, as in primary striatal neurons. We conclude that polyQ expansion beyond the disease threshold length leads to a steady but increased risk of cell death. These findings offer the first direct test and support of a recently proposed model of HD neurodegeneration inferred from pathological specimens³¹.

To examine IB formation and neuronal death, we first sought to confirm that we could detect and monitor IBs in live neurons. We previously reported that in cultured striatal neurons, polyQ-expanded Htt forms IBs that label with antibodies against ubiquitin⁵, as in HD. As with other cell types^{34,35}, some neurons containing polyQ-expanded Htt fused to GFP developed punctate, highly fluorescent intracellular structures resembling IBs (Fig. 1a, white and yellow arrows in bottom row; Fig. 2a). To characterize these structures further, we fixed GFP-tagged Htt *in situ* and measured its fluorescence before and after treatment with detergent²¹. GFP

fluorescence in the structures was not significantly affected, but was almost completely destroyed elsewhere in the neuron, indicating that these structures were possibly IBs (Fig. 2a, b).

Because the fluorescence intensity of Htt^{ex1}-GFP within IBs is almost fivefold that of diffuse Htt^{ex1}-GFP elsewhere in the neuron, we used this distinction to identify IBs within living neurons and to follow their fates longitudinally. IBs formed in neurons transfected with Htt^{ex1}-Q₄₇-GFP, Htt^{ex1}-Q₇₂-GFP or Htt^{ex1}-Q₁₀₃-GFP but not with Htt^{ex1}-Q₁₇-GFP or Htt^{ex1}-Q₂₅-GFP. IBs became detectable at less than 1 μm² and achieved sizes similar to those in HD^{2,3,36}, typically growing for as long as the neuron remained alive (Fig. 2c). Larger IBs are also more common in later stages of HD (ref. 36). Thus, the size and behaviour of IBs formed by Htt in transfected striatal neurons resemble those seen in HD.

Death without IB formation

If IBs trigger neuronal death through gradual sequestration and functional loss of other critical cellular proteins³⁷, functional loss of these critical proteins—and therefore the risk of death—should increase with the number and size of IBs (that is, the IB load). Over time, the size of IBs (Fig. 2c) and the fraction of neurons that contain them (that is, the prevalence; Fig. 2d) increased significantly. However, the risk of death from polyQ expansion is relatively constant (see above), indicating that IB load is unlikely to explain polyQ-dependent cell death.

Could an earlier form of polyQ-expanded Htt be the principal toxic species? To test this possibility we recorded the moment at which an IB was first detected (that is, the IB incidence) and measured its relationship to polyQ-dependent death. It has not been possible to measure IB incidence before because conventional approaches fail to record neurons that form IBs but die before they are detected and scored. IB incidence for Htt^{ex1}-Q₁₀₃-GFP was more

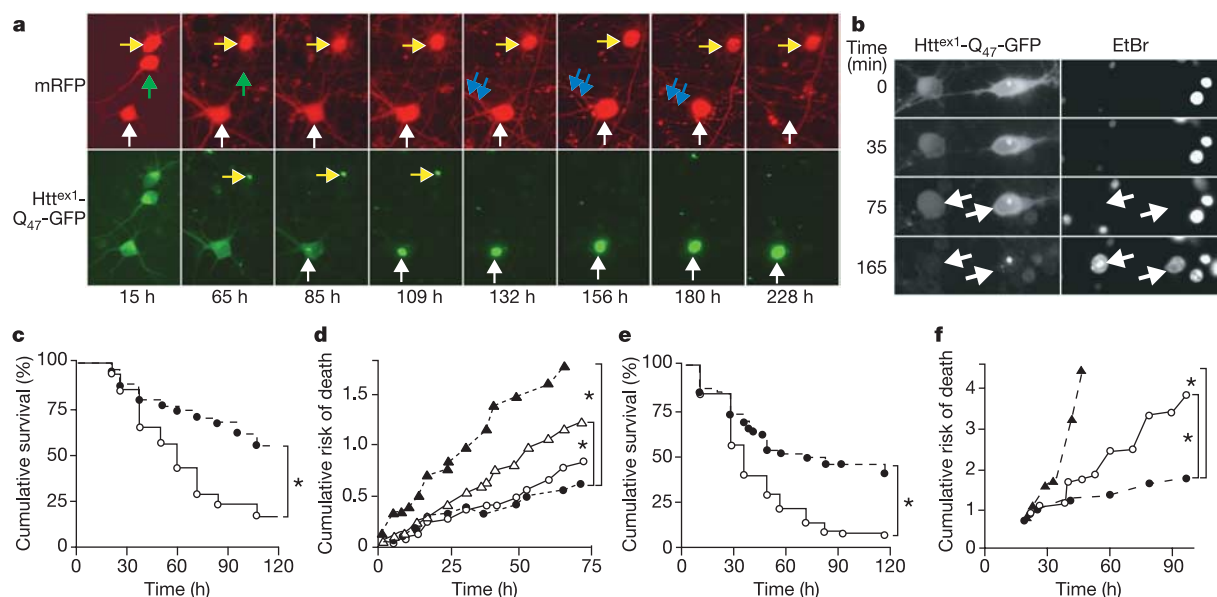


Figure 1 PolyQ-expansion-dependent cell death measured with an automated microscope. **a**, Longitudinal tracking of single neurons expressing mRFP (top panels) and Htt^{ex1}-Q₄₇-GFP (bottom panels). Two neurons (yellow and white arrows, top row) that formed IBs (yellow and white arrows, bottom row) outlived a third neuron, which died without an IB (green arrow). Soon after an IB formed (white arrow, bottom panel), mutant Htt disappeared elsewhere in the neuron. Neuron morphology remained intact for days (top row), but then neurites degenerated (blue arrows) and the neuron died. **b**, Abrupt loss of marker protein fluorescence (white arrows, compare two bottom left panels) is accompanied by staining with the nuclear dye ethidium bromide (EtBr), indicating death (white arrows; compare two bottom right panels). **c**, Survival analysis of neurons transfected with wild-type (Htt^{ex1}-Q₁₇-GFP, filled circles) or mutant (Htt^{ex1}-Q₇₂-GFP, open

circles) Htt illustrates polyQ-expansion-dependent death (*n* > 100 neurons, four experiments). **d**, Hazard analysis demonstrates that versions of Htt with disease-associated polyQ expansions increase the risk of death significantly and in a length-dependent fashion (*n* = 4). Filled triangles, Htt^{ex1}-Q₁₀₃-GFP; open triangles, Htt^{ex1}-Q₇₂-GFP; filled circles, Htt^{ex1}-Q₁₇-GFP; open circles, GFP. **e**, **f**, Homogeneous PC12 cells that are either stably (**e**) or transiently (**f**) transfected with Htt-GFP undergo a polyQ-expansion-dependent decrease in survival and corresponding increase in death risk (*n* > 200 PC12 cells, two or three experiments). Symbols in **e**: filled circles, Htt^{ex1}-Q₂₅-GFP; open circles, Htt^{ex1}-Q₁₀₃-GFP. Symbols in **f**: filled circles, Htt^{ex1}-Q₁₇-GFP; open circles, Htt^{ex1}-Q₄₇-GFP; filled triangles, Htt^{ex1}-Q₁₀₃-GFP. Asterisks in **c–f** indicate *P* < 0.0001.

than double that for Htt^{ex1}-Q₄₇-GFP (Fig. 2e). Expansion from 47 to 103 glutamine residues had a larger effect on the incidence (Fig. 2e) than on the prevalence of IBs (Fig. 2d). Importantly, the polyQ-expansion-dependent risk of death was better correlated with the initiation of IB formation than with IB load (Figs 1d and 2e; Supplementary Fig. S3). This finding indicates that the principal toxic species might be an early IB intermediate or a form of diffuse intracellular Htt.

Is IB formation even necessary for polyQ-expansion-dependent death? IB formation has been dissociated from polyQ-dependent death^{5,6,38,39}, but the lack of longitudinal, single-cell analysis and the potential nonspecific effects of exogenous manipulations left the interpretation of these experiments in doubt¹⁷. For example, if IB formation accelerates death, neurons might die too rapidly to be detected. However, experiments in which we collected images every 2 h showed that only 1% of neurons that formed an IB within a 24-h interval also died within that period. In fact, most neurons that form IBs can be followed for at least 2 days (Htt^{ex1}-Q₄₇-GFP, 71 ± 4%; Htt^{ex1}-Q₁₀₃-GFP, 55 ± 4% (±s.d.)). Thus, neurons that form IBs did not die too quickly for us to detect them. Moreover, survival analysis of Htt-transfected neurons that do not form IBs showed an increased risk of death among neurons transfected with Htt^{ex1}-Q₄₇-GFP or Htt^{ex1}-Q₁₀₃-GFP but not Htt^{ex1}-Q₁₇-GFP (Fig. 2f). These findings indicate that IB formation is not required for polyQ-expansion-dependent neuronal death and that other less aggregated or possibly monomeric species of polyQ-expanded Htt are toxic.

Levels of diffuse Htt govern survival

If the principal toxic species of Htt are distributed diffusely within neurons, their levels might be better predictors of neuronal death than IB formation. To determine whether GFP fluorescence can be used to quantify levels of GFP-tagged protein in single cells⁴⁰, we performed three experiments. Both population-based and single-cell approaches showed that GFP fluorescence predicted the levels of GFP or of Htt to which it was attached (Fig. 3a, b, Supplementary Fig. S4). We conclude that we can quantify the amount of Htt protein within living neurons by imaging the fluorescence of the GFP tag.

To determine the relationship between levels of Htt and neuronal longevity, we used Cox proportional hazard analysis of neurons transfected with Htt^{ex1}-Q₄₇-GFP. The Q₄₇ expansion is more typical among HD patients than Q₇₂ or Q₁₀₃. Htt^{ex1}-Q₄₇-GFP also leads to death more slowly than the longer expansions, increasing our ability to resolve relationships between its expression and survival or IB formation. Cox proportional hazard analysis was used because it can determine whether and to what extent levels of Htt at an early time point within individual neurons can predict the longevity of those same neurons. We measured fluorescence from diffuse Htt within neurons, excluding IB fluorescence because Htt within IBs might have a different bioactivity. The levels of diffuse Htt^{ex1}-Q₄₇-GFP in neurons on the first day after transfection were correlated significantly and negatively with lifespan (Fig. 3c). The amounts of GFP alone (Fig. 3d) or Htt^{ex1}-Q₁₇-GFP (not shown) were not predictive. To exclude the possibility that neuron-subtype differences in vulnerability were required for the relationship we observed, we performed similar experiments in the homogeneous PC12 cell line. As in neurons, levels of Htt^{ex1}-Q₄₇-GFP on the first day of survival analysis were a significant and negative predictor of survival, whereas the expression of the co-transfected marker protein, mRFP, had no predictive value (Fig. 3e). These results suggest that more diffuse forms of polyQ-expanded Htt are the principal toxic species and that their levels govern neuronal survival.

PolyQ expansions in ataxin-7 might cause toxicity by stabilizing ataxin-7, causing soluble forms to accumulate⁴¹. Could a similar effect explain how levels of diffuse polyQ-expanded Htt predict death? We measured the level of diffuse Htt in neurons before IBs

had formed to avoid potential confounding effects of IB formation on these measurements. In contrast to findings with ataxin-7 (ref. 41), polyQ expansion was correlated with lower levels of diffuse Htt^{ex1}-GFP (Fig. 3f); similar results have been reported in HD (ref. 42). Thus, the effects of polyQ expansion on the levels of Htt do not explain polyQ-expansion-dependent neuronal death. Rather, they indicate that the polyQ expansion confers toxicity on more diffuse forms of Htt independently of its overall effect on the number of Htt molecules.

IB formation prolongs survival

Correlations between polyQ expansion and IB formation or neuronal death could suggest that IBs are pathogenic. Indeed, the levels of diffuse Htt^{ex1}-Q₄₇-GFP on day 1 after transfection were

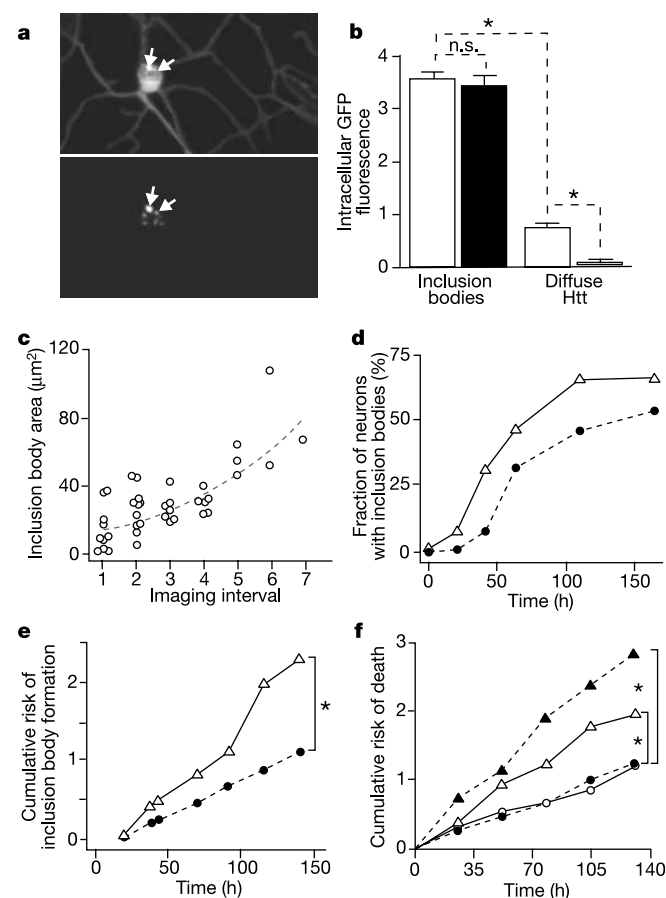


Figure 2 Many neurons die without forming IBs. **a**, Some neurons transfected with GFP-tagged versions of Htt with disease-associated polyQ expansions form highly fluorescent intracellular spheroid structures (arrows, top panel). Detergent treatment destroys GFP fluorescence except in these intracellular structures, indicating that they are IBs (arrows, bottom panel). **b**, Fluorescence intensity within IBs is very high, making it possible to monitor IBs in living neurons. A.u., arbitrary units of fluorescence intensity; n.s., not significant; $n = 10$ –541 neurons, three experiments. Open bars, in live neurons; filled bars, after detergent treatment. Error bars indicate s.e.m. **c**, IB growth was measured daily ($n = 12$). **d**, A cohort of neurons was monitored longitudinally. The fraction of neurons with IBs grows with time and is greater for those transfected with Htt^{ex1}-Q₁₀₃-GFP (triangles) than Htt^{ex1}-Q₄₇-GFP (circles) (three experiments). **e**, Cumulative risk of IB formation for Htt^{ex1}-Q₁₀₃-GFP (triangles) is about double that for Htt^{ex1}-Q₄₇-GFP (circles) and parallels the cumulative risk curves for survival ($n = 680$ neurons, three experiments). **f**, Neurons transfected with Htt that do not form detectable IBs nevertheless exhibit a significant polyQ-expansion-dependent increase in cumulative risk of death, indicating decreased survival ($n = 480$ neurons, three experiments). Filled triangles, Htt^{ex1}-Q₁₀₃-GFP; open triangles, Htt^{ex1}-Q₄₇-GFP; filled circles, Htt^{ex1}-Q₁₇-GFP; open circles, GFP. Asterisks in **b**, **e** and **f** represent $P < 0.0001$.

significantly and negatively correlated with the time of IB formation (Fig. 4a). Thus, levels of diffuse Htt^{ex1}-Q₄₇-GFP predict whether and when an IB forms and also longevity. However, the same patterns might be expected if IB formation were a cellular response to cope with more diffuse, toxic forms of Htt. By analysing images of neurons as they formed IBs, we found that levels of diffuse Htt-GFP elsewhere in the cell fell rapidly after an IB appeared (Fig. 1a, bottom row). Within a day or two, diffuse Htt was nearly undetectable (Fig. 4b), and the rapid decrease in diffuse GFP fluorescence was directly correlated with the rapid growth of the IB (Fig. 1a, white arrow in bottom row, compare 85 h with 109 h). In a few cases, several days after diffuse Htt was undetectable, the IB disappeared altogether (Fig. 1a, yellow arrow in bottom row).

For a direct investigation of the relationship between IB formation and the risk of death, we compared the survival curves of neurons that did or did not develop IBs. If IBs are pathogenic, neurons that develop them should die sooner than those that do

not. If IB formation is beneficial, the reverse might be true, and if IB formation is incidental, there might be no correlation with survival. To avoid selection bias, we identified all neurons that were alive at a particular time during the survival analysis and followed their fates prospectively. Neurons that contained or lacked an IB on the second day after transfection had similar risks of death (Fig. 4c).

However, on closer examination, we found that the subpopulation of neurons that form IBs on the second day also began with significantly higher intracellular levels of Htt-GFP (Fig. 4d). Thus, although the survival curves of the two populations were indistinguishable, the survival of neurons that formed IBs was better than that predicted by the relatively high initial expression of Htt-GFP (Fig. 3c). To test this idea further, we identified the

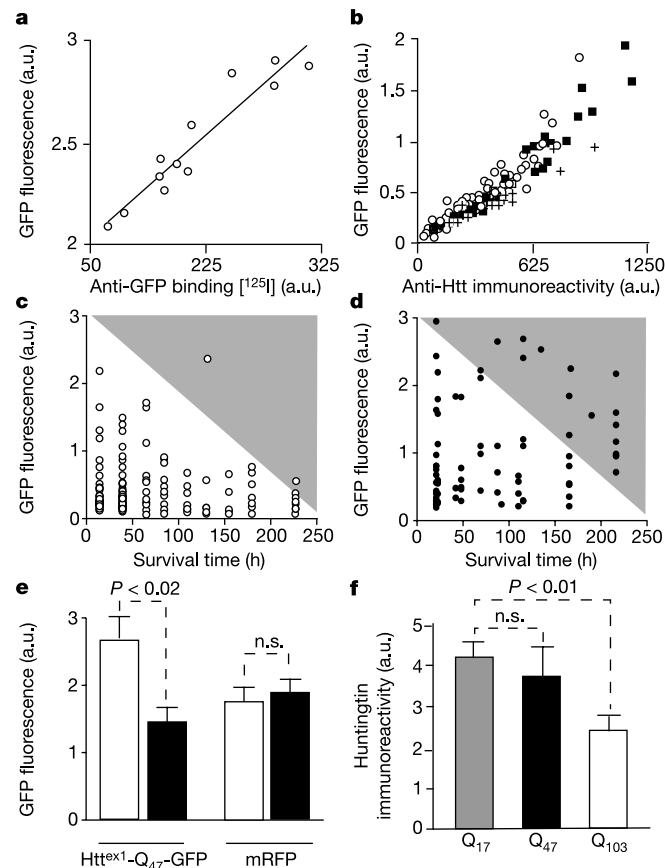


Figure 3 Levels of diffuse mutant Htt protein predict neuronal death. **a**, Cellular GFP fluorescence is well correlated with western blot measures of GFP within the same cells ($n = 2$): $r^2 = 0.9$; $P < 0.001$. **b**, Single-neuron levels of Htt fused to GFP estimated by imaging GFP fluorescence are well correlated with measurements by immunocytochemistry ($n = 2$). Open circles, Htt^{ex1}-Q₁₇-GFP ($r^2 = 0.8$); filled squares, Htt^{ex1}-Q₄₇-GFP ($r^2 = 0.9$); crosses, Htt^{ex1}-Q₁₀₃-GFP ($r^2 = 0.9$). **c**, Levels of diffuse Htt^{ex1}-Q₄₇-GFP are a significant ($P < 0.003$) and negative predictor of neuronal longevity. Fluorescence of diffuse Htt^{ex1}-Q₄₇-GFP was measured in individual neurons ($n = 217$ neurons, three experiments) on the first day after transfection and plotted against their respective survival times. **d**, Levels of GFP alone are not correlated with neuronal survival ($n = 97$ neurons, three experiments). **e**, Levels of Htt^{ex1}-Q₄₇-GFP but not the co-transfected marker, mRFP, are a significant and negative predictor of which PC12 cells live longer than 72 h ($n = 75$). Open bars, less than 24 h; filled bars, more than 72 h. **f**, Mean levels of Htt^{ex1}-GFP are significantly and negatively correlated with the length of the polyQ stretch within Htt^{ex1} ($n > 90$ neurons, three experiments). Error bars in **e** and **f** indicate s.e.m.

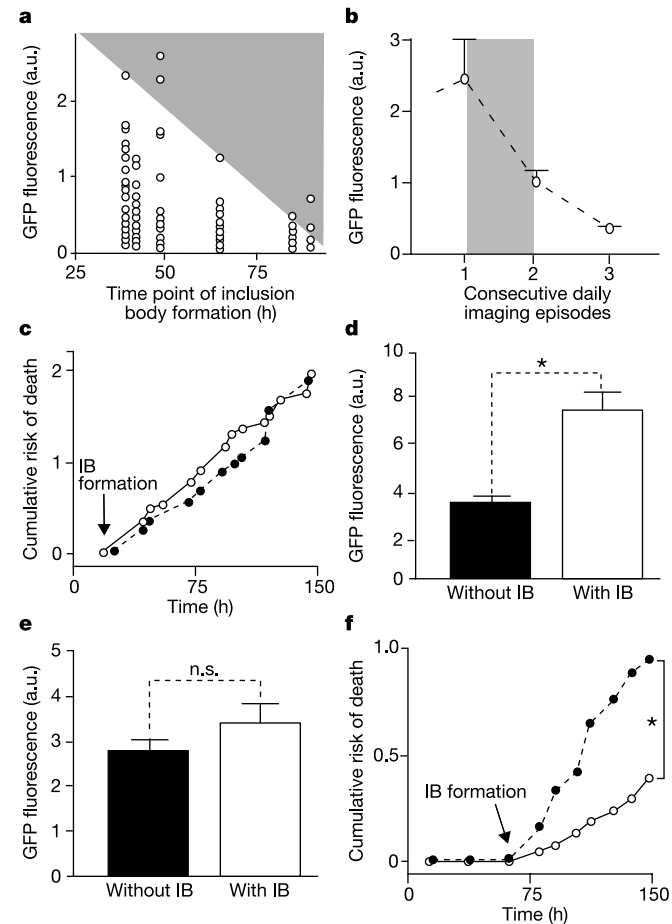


Figure 4 IB formation is associated with decreased intracellular levels of diffuse Htt^{ex1} and improved neuronal survival. **a**, Levels of diffuse Htt^{ex1}-Q₄₇-GFP are correlated ($P < 0.003$) with IB formation ($n = 105$, three experiments). **b**, GFP fluorescence within single neurons was measured over a region adjacent to the site of IB formation. Upon IB formation (area shown in grey), levels of Htt^{ex1}-Q₄₇-GFP elsewhere in the neuron decreased rapidly ($n = 10$). **c**, Neurons transfected with Htt^{ex1}-Q₄₇-GFP were divided into two cohorts depending on whether they contained an IB (open circles) or not (filled circles) on the second day they were imaged. The risk of death and the overall survival of neurons in these two cohorts were not significantly different ($n = 193$ neurons, three experiments). **d**, Neurons transfected with Htt^{ex1}-Q₄₇-GFP that contained an IB on the second day also began with significantly (asterisk, $P < 0.001$) higher levels of Htt^{ex1}-Q₄₇-GFP than the cohort of neurons without an IB on the second day. **e**, Neurons transfected with Htt^{ex1}-Q₄₇-GFP that formed IBs on the fourth day began with about the same levels of Htt^{ex1}-Q₄₇-GFP as the cohort of neurons that were alive on the fourth day but did not have IBs. **f**, IB formation is associated with reduced death risk and increased survival among neurons transfected with Htt^{ex1}-Q₄₇-GFP that are alive beginning on the fourth day ($n = 224$ neurons, three experiments). Open circles, with an IB; filled circles, without an IB. Asterisk, $P < 0.0003$. Error bars in **b**, **d** and **e** indicate s.e.m.

subpopulations of living neurons that either did or did not form an IB on the second day and that had similar initial levels of Htt-GFP. Prospective analysis revealed that neurons that formed IBs on the second day survived significantly longer than adjacent neurons that did not (Supplementary Fig. S5).

To further distinguish the contributions of Htt-GFP expression and IB formation to neuronal survival, we compared subpopulations of neurons with more closely matched levels of Htt-GFP. On either the fourth or sixth day after transfection, all living neurons started with similar levels of Htt-GFP, irrespective of whether they had developed an IB (Fig. 4e). We followed the survival of each of these populations prospectively. Neurons that formed an IB on either the fourth or sixth day survived significantly longer than adjacent neurons, which were otherwise similar but without an IB. IB formation was associated with a decrease in the cumulative risk of death (Fig. 4f, Supplementary Figs S6 and S7) to that seen with wild-type Htt (Htt^{ex1}-Q₁₇-GFP, data not shown). Moreover, PC12 cells that formed IBs survived significantly longer than those that did not, indicating that neuron-subtype differences in IB formation and viability were not required for the relationship we observed (Supplementary Fig. S8). IB formation was generally associated with a decrease in more diffuse forms of intracellular Htt and a corresponding improvement in survival.

In our cellular model, IBs form in the cytoplasm and in the nucleus, as in HD. The nucleus seems to be an important site of toxicity for mutant Htt^{5,43,44}. IBs could therefore be pathogenic in one location and beneficial in another. Analysis of neurons with cytoplasmic or nuclear IBs showed similar survival curves for both populations, and both survived significantly longer than neurons without IBs (data not shown). Thus, IB formation predicted increased survival regardless of the subcellular location.

Discussion

Using survival analysis, we found that neurons die from Htt protein in a manner best predicted by the level of diffuse forms of Htt and by the length of their polyQ expansions. PolyQ expansion increased the risk of death independently of its effect on the intracellular level of diffuse Htt. Surprisingly, IB formation reduced intracellular levels of diffuse Htt and prolonged survival. Together, these findings indicate that IB formation might protect neurons by decreasing the levels of toxic diffuse forms of mutant Htt (Fig. 5). The model is consistent with observations from post-mortem HD tissue, which reveal that IBs were more frequent in subpopulations of neurons that disproportionately survived³⁸. It remains unclear whether levels fall because of autophagy⁴⁵, because IBs sequester diffuse Htt or because IB formation is part of an adaptive programme that promotes increased Htt turnover⁸. Our results support the hypothesis that manipulations that improve survival and decrease IB formation (for example, certain aggregation inhibitors) might do so by interfering with the formation of toxic diffuse Htt species or with their ability to act on critical intracellular cellular targets^{46–48}. Although our data do not exclude a non-cell-autonomous role for IBs in pathogenesis, the appearance of IBs in unrelated neurodegenerative diseases, such as Parkinson's disease and HD, might reflect a common coping response by neurons to diffuse toxic protein instead of a common pathogenic mechanism¹².

In this study, survival analysis was essential for explaining the complex relationships between Htt expression, IB formation and

neuronal death. This approach might prove particularly useful for disease-related research in which intermediate cellular and histological abnormalities can be clearly defined but whose precise relationship to pathogenesis can be obscure. Determining whether a particular change is pathogenic, incidental or beneficial has important implications for understanding mechanisms of disease and for identifying therapeutic targets. When pathogenesis is mediated by multiple effectors, survival analysis provides a way to quantify the contribution of each factor, potentially helping to assess its individual pathogenic significance. □

Methods

Plasmids

Expression plasmids encoding an N-terminal fragment of Htt fused to GFP (pGW1-Htt^{ex1}-Q₂₅, Q₄₇, Q₇₂ or Q₁₀₃)-GFP) were derived from pcDNA3.1-based plasmids²¹ by subcloning into pGW1-CMV (British Biotechnologies). A PCR product of exon 1 of human Htt with 17 CAG repeats was ligated to GFP and used to create pGW1-Htt^{ex1}-Q₁₇-GFP. A PCR product of *mRFP1* was ligated into pGW1-CMV to create pGW1-mRFP and into pcDNA3.1(+) to create pcDNA3.1-mRFP. Plasmid constructions were confirmed by DNA sequencing.

Cell culture and transfection

Primary cultures of rat striatal neurons were prepared from embryos (embryonic days 16–18) and transfected with plasmids (6–7 days *in vitro*) as described^{5,49} (<http://gweb1.ucsf.edu/labs/finkbeiner>). Typically, neurons were co-transfected with pGW1-mRFP and a version of pGW1-Htt^{ex1}-[Q₁₇, Q₂₅, Q₄₇, Q₇₂ or Q₁₀₃]-GFP in a 1:1 molar ratio, using a total of 1–4 µg of DNA in each well of a 24-well plate. After transfection, neurons were maintained in serum-free medium.

To perform a modified LIVE-DEAD assay (Molecular Probes), growth medium was replaced with Eagle's basal medium 48 h after transfection. At 20 min before treatment with kainate (Sigma), ethidium homodimer (5 µM; Molecular Probes) was added, and images of transfected neurons were collected before and every 30 min after kainate addition. A detergent-resistance assay was performed as described²¹, with minor modifications. Neurons with putative IBs were imaged, treated with 1% paraformaldehyde for 15 min at 37 °C followed by 5% Triton X-100 and 5% SDS for 20 min at 37 °C, and imaged again. PC12 cells inducibly expressing Htt^{ex1}-Q₂₅-GFP or Htt^{ex1}-Q₁₀₃-GFP⁵⁰ were plated at 10⁴ cells per cm², transiently transfected with pcDNA3.1-mRFP and induced with 1 µM tetracycline. In some experiments, wild-type PC12 cells were plated at 5 × 10⁴ cells per cm² and co-transfected with a version of pGW1-Htt^{ex1}-[Q₁₇, Q₂₅, Q₄₇, Q₇₂ or Q₁₀₃]-GFP and pcDNA3.1-mRFP in a 1:1 molar ratio, using a total of 2 µg of DNA in each well of a 24-well plate.

Immunocytochemistry

Striatal neurons grown on 12-mm glass coverslips were examined 36 h after transfection as described⁵, with anti-GFP (1:500 dilution; Chemicon), anti-Htt EM48 (1:50 dilution; Chemicon) and anti-chicken or anti-rabbit Cy3-labelled antibodies (1:300 dilution; Jackson Immunochemical).

Western blots

HEK-293 cells grown in DMEM medium containing 10% calf serum, 2 mM glutamine and penicillin/streptomycin (100 U ml⁻¹/100 µg ml⁻¹) were transiently transfected with pGW1-GFP (1–6 µg per well). Images were captured every 24 h for 3 days. Protein extracts were prepared from cells immediately after imaging, subjected to SDS-polyacrylamide-gel electrophoresis, blotted with anti-GFP antibody (1:1000 dilution; Zymed) and detected with ¹²⁵I-labelled secondary antibody and a PhosphorImager screen (Fuji).

Robotic microscope imaging system

The system is based on an inverted Nikon microscope (TE300 Quantum). Olympus 4 × (numerical aperture 0.13) and 10 × (numerical aperture 0.30) and Nikon 20 × (numerical aperture 0.45) objectives were used. Xenon lamp (175 W) illumination was supplied by a liquid light guide to reduce electrical noise. Images were detected and digitized with a Hamamatsu Orca II 12/14-bit, digital, cooled charge-coupled device camera and Universal Imaging Metamorph software. Stage movements and focusing were executed with computer-controlled stepper motors. Fluorescence excitation and emission filters were moved into or out of the optical path with each program loop by two ten-position filter wheels (Sutter Instruments) under computer control. The whole system is mounted on a vibration isolation table to reduce noise. Computer commands that perform and coordinate automated stage movements, filter wheel movements and focusing were generated with software programs that combine custom-designed and commercially available algorithms. Additional programs for image analysis were written with MatLab and Visual C software.

Image and statistical analysis

Measurements of Htt expression, IB formation and neuron survival were extracted from files generated with automated imaging by automated analysis programs or by visual inspection. Automated programs identified living transfected neurons by physical dimensions and fluorescence. IBs were monitored by size and fluorescence intensity. The expression of GFP-tagged versions of Htt was estimated by measuring GFP fluorescence intensity over a region of interest that corresponded to the cell soma or as otherwise

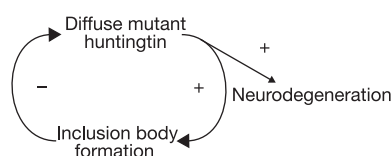


Figure 5 A model of the role of IB formation in huntingtin-induced neurodegeneration.

indicated, using the fluorescence of co-transfected mRFP as a guide. These GFP intensity values were background-subtracted by using an adjacent area of the image.

For statistical analysis, survival time was defined as the imaging time point at which a cell was last seen alive. Kaplan–Meier curves were used to estimate survival and hazard functions with commercially available software (Statview). Differences in Kaplan–Meier curves were assessed with the log-rank test. Linear regression was used to correlate Htt expression measured with different methods, and correlations between Htt expression and survival or IB formation were made with Cox proportional hazard analysis. Differences in mean measurements were compared by analysis of variance or *t*-test.

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Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*

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Glutamate transporters are integral membrane proteins that catalyse the concentrative uptake of glutamate from the synapse to intracellular spaces by harnessing pre-existing ion gradients. In the central nervous system glutamate transporters are essential for normal development and function, and are implicated in stroke, epilepsy and neurodegenerative diseases. Here we present the crystal structure of a eukaryotic glutamate transporter homologue from *Pyrococcus horikoshii*. The transporter is a bowl-shaped trimer with a solvent-filled extracellular basin extending halfway across the membrane bilayer. At the bottom of the basin are three independent binding sites, each cradled by two helical hairpins, reaching from opposite sides of the membrane. We propose that transport of glutamate is achieved by movements of the hairpins that allow alternating access to either side of the membrane.

The chemical synapse is a central site for communication between neurons in the human brain. At chemical synapses an action potential promotes the release of neurotransmitter, increasing the concentration of transmitter 10^3 – 10^4 -fold in the synaptic cleft. The neurotransmitter opens ligand-gated ion channels, resulting in depolarization of the postsynaptic neuron and generation of a postsynaptic receptor potential. At many synapses, integral membrane transport proteins clear the transmitter from the synaptic cleft, reducing the concentration of transmitter to basal level, thereby readying the synapse for a subsequent cycle of activation¹.

Glutamatergic synapses are the chemical synapses that mediate the majority of fast excitatory neurotransmission². Essential for normal development and function, the glutamatergic synapse is a linchpin for learning and memory, and dysfunction at these synapses is implicated in a wide range of nervous system diseases and injuries, including schizophrenia, depression and stroke³. Rapid clearance of glutamate from the synapse by high-affinity, sodium-dependent transporters is required for normal excitatory neurotransmission and prevention of glutamate-induced excitotoxicity^{4,5}.

The high-affinity, sodium-dependent glutamate transporters are members of a family of integral membrane transport proteins that include five eukaryotic glutamate transporters, two eukaryotic neutral amino acid transporters, and a large number of bacterial amino acid and dicarboxylic acid transporters^{5,6}. Eukaryotic members of this transporter family have an essential role in the nervous system and they function in many other organs, including the heart, kidney and intestine⁷. In prokaryotes, these transporters carry out the concentrative uptake of metabolites across the membrane by the co-transport of protons and/or sodium ions⁶. Physiological studies have elaborated the ion stoichiometry of eukaryotic glutamate transporters, showing that glutamate uptake is coupled to the co-transport of three sodium ions and one proton, and to the counter-transport of one potassium ion⁸. Notably, eukaryotic glutamate transporters also possess a thermodynamically uncoupled, glutamate-gated chloride conductance, illuminating their dual roles as secondary transporters and ligand-gated ion channels⁹.

Prokaryotic and eukaryotic glutamate and neutral amino acid transporters possess significant amino acid sequence relationships throughout their entire polypeptides⁶ (Fig. 1). Residues in the carboxy-terminal half of eukaryotic and prokaryotic transporters are crucial for substrate binding, substrate transport and ion

coupling (for recent reviews, see refs 10–12), whereas residues in the amino-terminal portion of the eukaryotic transporters are implicated in the thermodynamically uncoupled chloride flux¹³. Determination of the transmembrane topology of glutamate transporters has been fraught with uncertainty, and there are multiple models, each possessing non-canonical elements of transmembrane protein structure^{14–20}. Thus, despite the wealth of functional data on glutamate transporters, there is no understanding of their three-dimensional architecture or molecular transport mechanism.

Structure determination

To reveal the molecular architecture of glutamate transporters, and to provide an atomic basis for a mechanism of substrate and ion transport, we crystallized a glutamate transporter homologue from *P. horikoshii* (Glt_{ph}; Supplementary Table S1), which shares 37% amino acid identity with human excitatory amino acid transporter 2 (hEAAT2). In the course of our crystallization trials we found that a multiple point mutant of Glt_{ph}, in which seven His residues were introduced into non-conserved sites on predicted loops (Glt_{ph}7H), was expressed at higher levels and crystallized more readily than the wild-type protein (Fig. 1).

Crystals of Glt_{ph}7H diffract to 3.2 Å along *c** and 3.8 Å along *a** and belong to the space group *P*6₁ (Table 1). Initial phases were obtained from a 6 Å resolution multiwavelength anomalous diffraction (MAD) experiment²¹ using a platinum derivative. Six heavy atom sites, arranged as two sites per protomer, confirmed the trimeric subunit stoichiometry of prokaryotic transporters²² and defined a three-fold axis of non-crystallographic symmetry (NCS). The MAD phases were applied to a native data set and extended to 3.5 Å resolution using DM²³. To assist in model building we exploited the presence of 16 Met residues per protomer by determining selenium sites from anomalous difference Fourier maps of a selenomethionine derivative. In addition, we substituted Met residues into six sites with ultimately each transmembrane segment containing one or two Met residues (Fig. 1; see also Supplementary Table S2). Iterative cycles of model building and refinement were then carried out. The final model contains all amino acid residues except for 11 N-terminal and 6 C-terminal residues, and a number of disordered side chains modelled as Ala.

We also collected data from an isomorphous crystal of the wild-type Glt_{ph} protein that diffracted to 4.1 Å (Supplementary Table S2). The phases from Glt_{ph}7H were applied to the Glt_{ph} data, followed by density modification and crystallographic refinement. The partially

refined structure and accompanying electron density maps did not reveal any significant differences between Glt_{ph}H7 and Glt_{ph}.

Trimer architecture

The Glt_{ph}H7 trimer is bowl-shaped with a concave aqueous basin facing the extracellular solution and a pointed base facing the cytoplasm (Fig. 2). The three-fold NCS axis is perpendicular to the membrane, and when viewed in this orientation the trimer has a triangular shape with sides of ~80 Å. Viewed parallel to the membrane, the trimer is ~65 Å in height, with the transmembrane-spanning portion of the transporter lying approximately in the middle, thus indicating that the transporter protrudes about 15 Å from each side of the membrane bilayer. The basin is as large as 50 Å in diameter and 30 Å in depth, and dips far into the membrane plane. Because the extracellular basin is deep and its surface hydrophilic, it allows aqueous, bulk solution to reach the midpoint of the membrane bilayer (Fig. 2d).

There are prominent crevices between the subunits on the lipid-exposed surface of the trimer (Fig. 2). Transmembrane 4 (TM4) is located in this crevice and participates in intersubunit contacts. TM1 and TM6 form an additional crevice on the lipid-exposed face of each subunit. In electron density maps we see non-protein density features in both crevices that may be bound lipid or detergent molecules. These crevices allow lipid molecules to access helical hairpins 1 and 2 (HP1, HP2), which are key functional regions of the transporter, and may provide a structural basis for understanding how lipids modulate the activity of bacterial and eukaryotic transporters^{24–26}.

Protomer structure

Glt_{ph}H7 protomers are shaped like pointed wedges where the wide ends define the extracellular rim of the basin and the pointed tips come together at the three-fold axis, forming the bottom of the basin and, on the intracellular face, a cone-shaped structure.

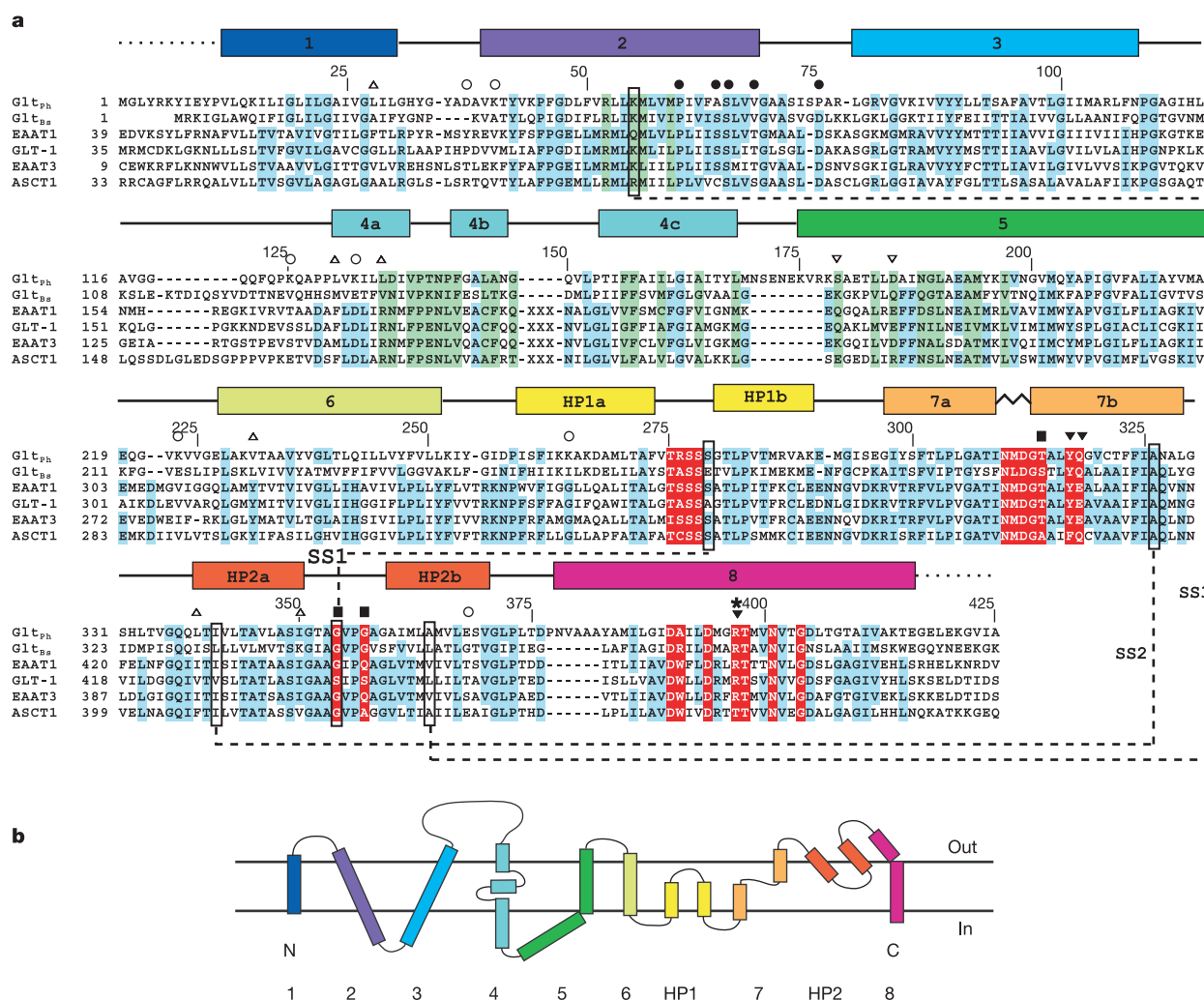


Figure 1 Sequence alignment of Glt_{ph}, glutamate and neutral amino acid transporters. **a**, Boxes above the alignment correspond to α -helices and are colour-coded according to Fig. 3. Dotted lines represent residues disordered in the crystal structure. Sequence colouring highlights regions of high homology (blue), intersubunit contacts seen in the crystal structure (green) and residues implicated in glutamate transport (red). Filled symbols above the sequences mark residues involved in glutamate γ -carboxylate binding (star), sodium binding (squares), potassium coupling (inverted triangles) and chloride conductance (circles). Open symbols mark the histidine point mutants (circles), the methionine mutants (triangles) and the double cysteine mutant (inverted triangles).

Residues in eukaryotic transporters that form disulphide bonds when mutated to cysteines are boxed and the bonds are indicated by dashed lines^{13,29}. Insertions in eukaryotic transporters between helices 4b and 4c are not included and are marked by XXX; the longer N and C termini of eukaryotic transporters are also not included. Amino acid sequences are: *P. horikoshii* Glt_{ph} (NP_143181), *B. stearothermophilus* Glt_{ss} (P24943); human EAAT1 (P43003); rat GLT-1 (P31596); human EAAT3 (AAH37310); human ASCT1 (NP_003029). The alignment was made using ClustalW⁵⁰ and adjusted manually.

b, Schematic representation of Glt_{ph} transmembrane topology.

Table 1 Diffraction data and refinement statistics

Data set	Native	Pt (peak)	Pt (inflection)	Pt (remote)	Se (peak)
Data source	BNL X25	BNL X6A	BNL X6A	BNL X6A	BNL X25
Wavelength (Å)	1.10	1.0721	1.0711	0.9918	0.9791
Cell (a × c) (Å)	116 × 322.3	112.4 × 320	112.4 × 320	112.6 × 320.4	115.2 × 323.6
Resolution (Å)	34–3.5	35–6	35–6	35–6	34–3.8
Completeness (%) [*]	99 (98)	97.8 (86.5)	98.4 (91.7)	99.2 (97.0)	95 (95)
Redundancy	6.7 (4.4)	3.5 (1.6)	3.7 (2.4)	3.5 (3.0)	5.5 (4.4)
R_{merge} (%) [†]	7.8 (84)	4.0 (42)	3.5 (38)	4.0 (47)	9.7 (55)
I/σ	7.3 (0.7)	35 (2.0)	37 (2.3)	34 (2.2)	4.8 (1.4)
Unique reflections	30,587	11,109	11,116	11,271	21,550
Phasing					
R_{cullis}		0.645	0.76	0.77	
Phasing power [‡]		1.96	1.40	1.37	
Overall figure of merit		0.524			
NCS correlation (%) [§]	83.4				
Refinement statistics			Model geometry		
Resolution range (Å)	10–3.5		r.m.s.d. (angle/bond)		1.55°/0.012 Å
$R_{\text{work}}/R_{\text{free}}$ (%)	29.0/30.9		Ramachandran plot		
Number of reflections	27,199		Favoured (%)		79.2
Completeness (%)	97.1		Allowed (%)		20.0
Number of atoms	8,658		Generously allowed (%)		0.8
Average B-factor (Å ²)	124.4		Disallowed (%)		0.0

^{*}Values for the highest resolution shell are shown in parentheses and the shells are 3.7–3.5 Å, 6.2–6.0 Å and 4.0–3.8 Å for the native, Pt and Se data sets, respectively.

[†] $R_{\text{merge}} = \sum |I_{\text{hkl}} - \langle I_{\text{hkl}} \rangle| / \sum I_{\text{hkl}}$, where I_{hkl} is the integrated intensity of the given reflection.

[‡]Phasing power, $\sum |F_{\text{hkl}}| / \sum |E|$, was calculated using SHARP.

[§]Correlation between the electron density areas related by non-crystallographic symmetry.

$R_{\text{work}} = (\sum |F_{\text{o}} - F_{\text{c}}|) / \sum F_{\text{o}}$, where F_{o} and F_{c} are observed and calculated structure factors.

[¶]Five per cent of data were excluded from the refinement to calculate R_{free} .

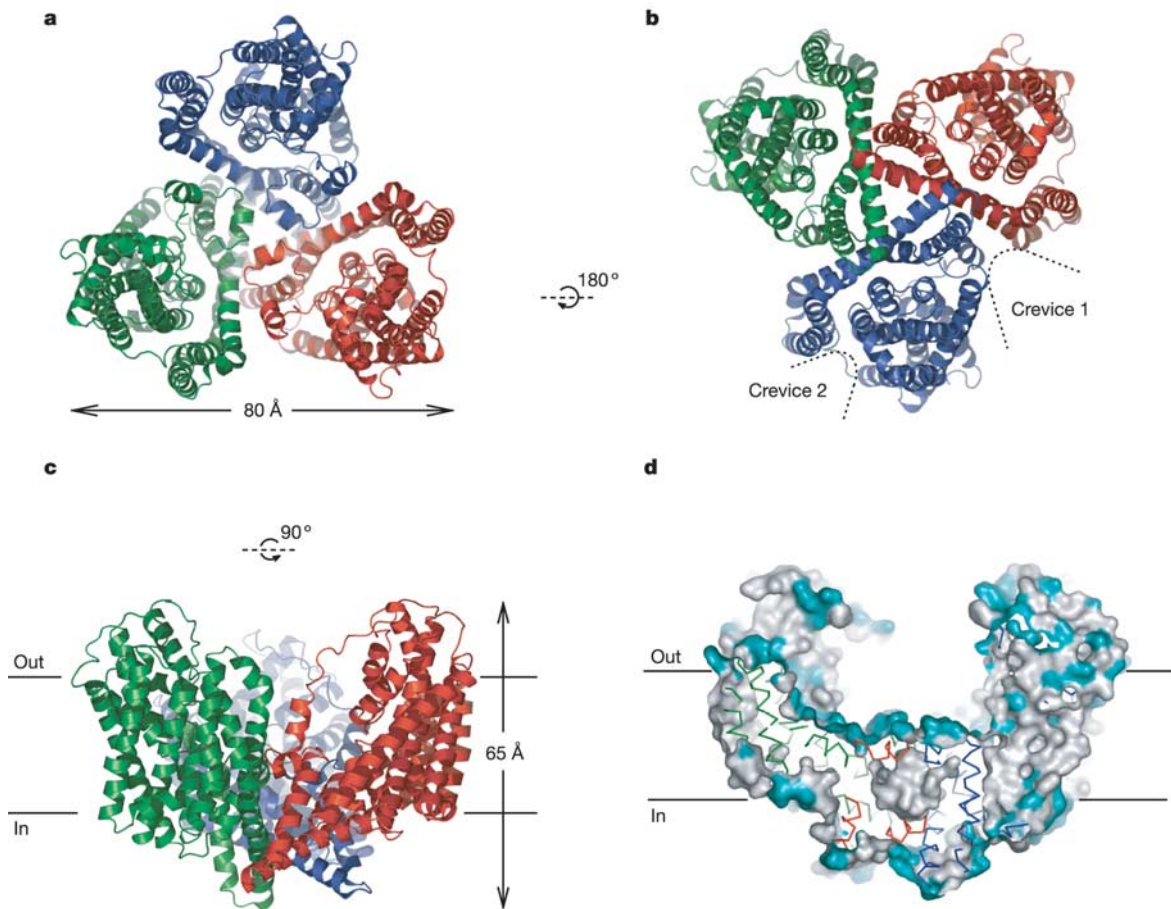


Figure 2 Structure of Glt_{P^H}H7. **a**, Ribbon representation of the trimer, in which the protomers are red, blue and green, viewed from the extracellular side of the membrane. **b**, View of the trimer from the cytoplasm, showing the locations of crevice 1, between subunits, and crevice 2, between transmembranes 1 and 6 of each subunit. **c**, View of the

trimer parallel to the membrane. **d**, Surface representation of the trimer sliced through the centre of the basin. Polar and apolar residues are coloured cyan and white, respectively. The boundaries of the lipid bilayer are indicated in **c** and **d**, using the hydrophobic residues on TM1 as a reference.

Each protomer has eight primarily α -helical transmembrane segments (TMs 1–8) and two helical hairpins (HPs 1–2; Figs 1 and 3). Transmembrane segments 1–6 form a distorted cylinder-shaped motif—the N-terminal cylinder—whose outer surface mediates all of the intersubunit contacts in the trimer. The C-terminal half of the protein—TM7, TM8, HP1 and HP2, implicated in substrate transport—is secured within the N-terminal cylinder, suggesting that each subunit has an independent substrate transport pathway.

The fold of a Glt_{ph}7H protomer is, to the best of our knowledge, novel and is composed of a number of unusual elements of secondary structure. In particular, TM2, TM3 and TM5 are up to 49 residues in length and are tilted from the membrane normal by as

much as 45°. The long, protease-sensitive and proline-rich ‘linker’ that connects TM3 and TM4 (refs 22, 27) arches from one side of the N-terminal cylinder to the other, over the top of HP2 and TM7 and TM8, spans a distance of about 60 Å, and makes only a few contacts with other portions of the subunit. TM4 is composed of multiple elements, has a corkscrew-like, helix-turn-helix-turn-helix structure and forms key subunit–subunit contacts on the three-fold axis.

The C-terminal portion of the protomer includes essential elements of the transport machinery. Helical hairpin 1 (HP1) is a helix-turn-helix structure that begins on the cytoplasmic surface of the trimer and is buried within the N-terminal cylinder, reaching up

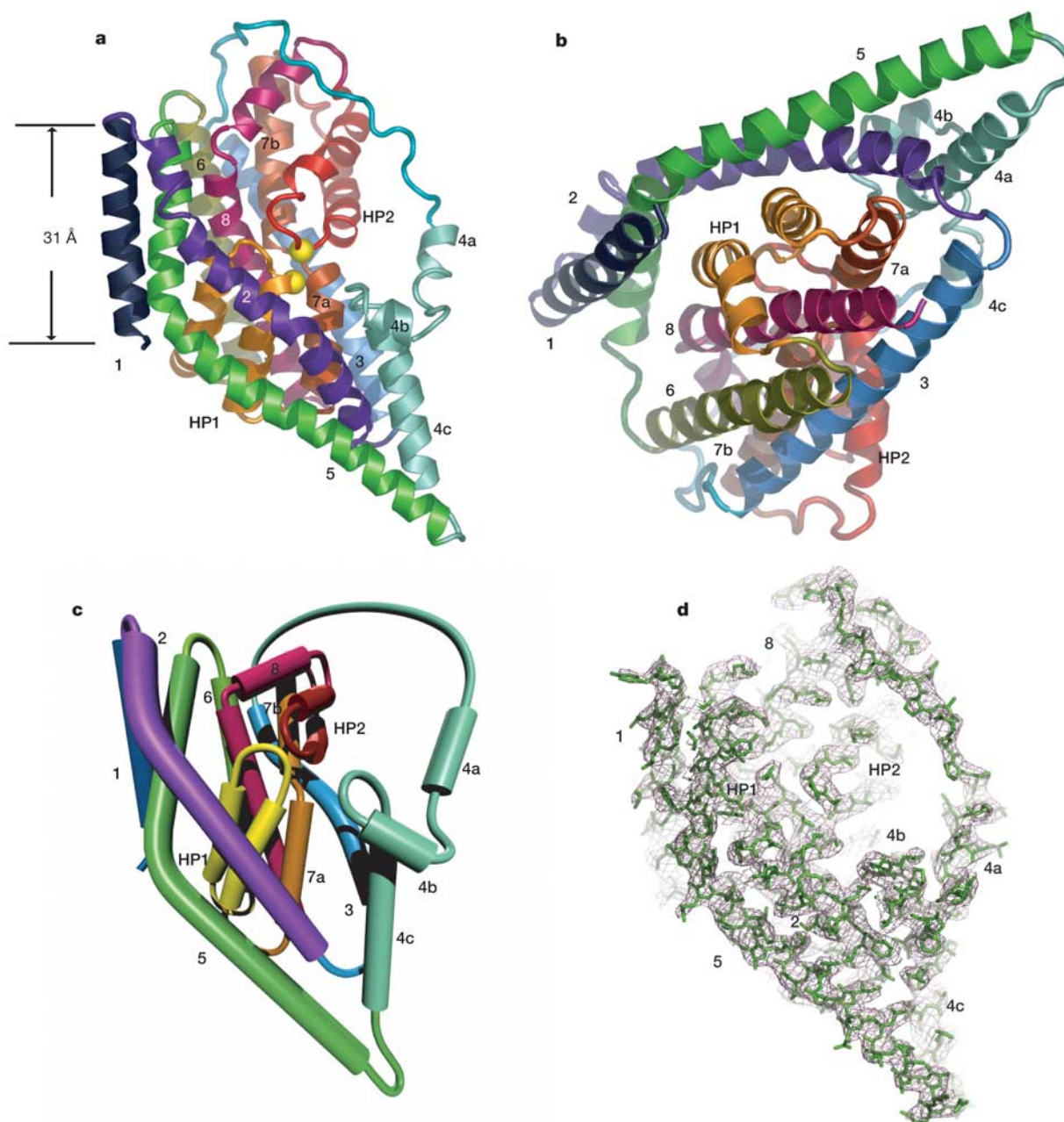


Figure 3 Fold of a Glt_{ph}7H protomer. **a**, Ribbon representation of the protomer viewed in the plane of the membrane in which the transmembrane helices (1–8) and hairpins (HP1, HP2) are labelled and in different colours. The α -carbon atoms of Ser 279 (HP1) and Gly 354 (HP2) are defined by yellow spheres, which are equivalent to Ala 364 and Ser 440

of GLT-1 (ref. 29). **b**, View of the protomer from the cytoplasm. **c**, Schematic representation of the protomer fold. **d**, Slice of electron density from a 2F_o - F_c map, contoured at 1 σ , overlaying a stick model of a protomer.

to the bottom of the extracellular basin. A conserved serine-rich motif located in the loop of HP1 tiles part of the basin bottom and is partially exposed to the extracellular solution, in agreement with previous chemical modification experiments^{14,16,18}. Passing through the middle of the N-terminal cylinder is TM7, an unusual transmembrane structure with two helical segments, 7a and 7b, whose helical axes are parallel but displaced by a conserved, three-residue motif that forms a β -bridge.

Helical hairpin 2 (HP2) is another key element of the transport machinery and like HP1 it is composed of a helix-turn-helix motif. However, the context of HP2 is different; it is situated almost parallel to the membrane plane, with a large fraction of its surface solvent-exposed and facing the extracellular basin. At the tip of HP2 there is a conserved proline (Pro 356 in Glt_{ph}7H) in van der Waals contact with the serine-rich motif at the tip of HP1. Connected to HP2 is TM8, an amphipathic α -helical segment that runs through the middle of the N-terminal cylinder and has been suggested to line a portion of the substrate transport pathway²⁸.

HP1 and HP2, together with flanking regions from TM7 and TM8, are structurally related and can be superimposed with a root mean square deviation (r.m.s.d.) of 2.4 Å (Supplementary Fig. S1), even though HP1 and HP2 have no significant amino acid sequence identity. Most importantly, the tips of HP1 and HP2 meet at the bottom of the basin, about halfway across the membrane bilayer. The apposition of HP1 and HP2 was foreshadowed by experiments on the rat glutamate transporter GLT-1, in which Ala 364 and Ser 440 were changed to cysteine. This double cysteine mutant of GLT-1 was active in glutamate transport only under reducing conditions, suggesting that a disulphide bond formed between residues 364 and 440 under oxidizing conditions²⁹. In Glt_{ph}7H the residues equivalent to Ala 364 and Ser 440 of GLT-1 are Ser 279 and Gly 354, respectively, they map to the tips of HP1 and HP2 and are sufficiently close to form a disulphide bond (Fig. 3a; see also Supplementary Fig. S1).

Subunit interface and oligomerization state

The Glt_{ph}7H protomers share substantial intersubunit interfaces with each subunit burying $\sim 2,045 \text{ Å}^2$ in a trimerization motif composed of TM2, TM4 and TM5. On the cytoplasmic face of Glt_{ph}7H the C-terminal portion of TM4c and the N-terminal end of TM5 form a bundle crossing or a 'smoke-hole' (Figs 2b and 4a). On the extracellular side of the transporter the TM4b helices define a whorl around the symmetry axis (Fig. 2a) while TM2 cements intersubunit contacts between TM4c/TM5 in one subunit and the corkscrew/TM4c of its neighbour. Eukaryotic transporters have insertions of 32–55 amino acids between TM4b and TM4c (Fig. 1), which may be accommodated within the basin.

Viewed along the membrane normal TM4b, TM4c, TM5 and TM2 form a distinct trimerization domain (Figs 2a, b and 4). At the centre of the domain, around the three-fold axis, is a vestibule of $\sim 400 \text{ Å}^3$ (Fig. 2d). The residues lining the vestibule are hydrophobic and even though there are positive electron density features in the cavity, identification of the chemical composition of the bound molecule(s) is not possible at this moderate resolution. There are small portals into the vestibule, from both the basin and the cytoplasmic smoke-hole, but the diameters of the openings are only 2–3 Å. Given its nonpolar character and limited access, the vestibule is unlikely to serve as a permeation pathway for ions or substrates.

To confirm our assignment of key subunit–subunit contacts, we designed a double cysteine mutant (Ser 179 changed to Cys/Asp 185 changed to Cys, referred to hereafter as S179C/D185C) to form an intersubunit disulphide bond linking subunits together (Fig. 4a). The double cysteine mutant, when treated with copper phenanthroline, forms a 138-kDa trimer, as determined by mass spectrometry (Fig. 4b). Because these two non-native cysteine residues readily form a disulphide-linked trimer, our crystal structure is relevant to

the oligomerization state of the transporter in a non-crystalline environment.

To determine the subunit stoichiometry of eukaryotic transporters we expressed the human EAAT2 transporter in HEK 293 cells as a fusion with green fluorescent protein (hEAAT2–GFP). After purification by size exclusion chromatography and glutaraldehyde cross-linking, hEAAT2–GFP forms a pattern of cross-linked species that is consistent with a trimer (Fig. 4c). Therefore, on the basis of the Glt_{ph}7H structure, the conservation of residues in subunit interfaces, the cross-linking of hEAAT2–GFP and previous work from this group²² and others³⁰, proteins in the prokaryotic and eukaryotic glutamate transporter family are trimers.

Substrate-binding site

A telltale clue to the binding site for substrate along the transport pathway comes from conspicuous non-protein electron density near the interface between HP1 and HP2 (Fig. 5). This electron density feature, approximately the size of a glutamate molecule, cannot be modelled as a protein side chain, and after real space three-fold averaging is greater than 6σ . Because we included L-glutamate at all stages of purification and crystallization, it is possible that the electron density is a glutamate molecule. However, owing to the modest resolution of our diffraction data we cannot unambiguously identify the molecule(s). We also have been unable to elicit transport activity from Glt_{ph}, using a number of methods, suggesting that Glt_{ph} may require archaeal lipids or an elevated

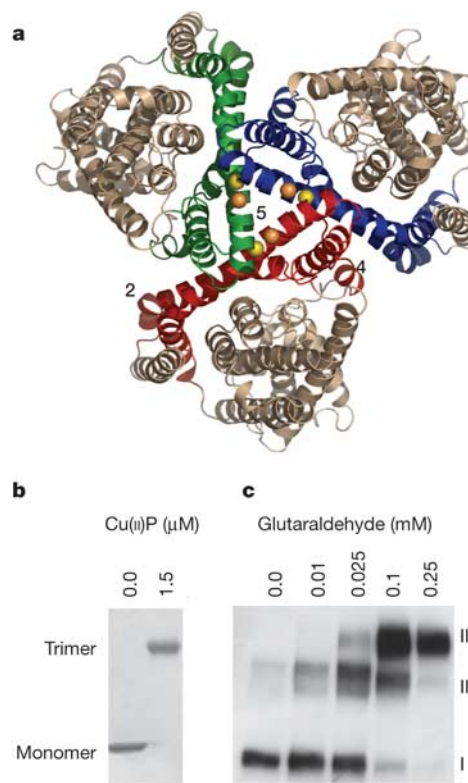


Figure 4 Oligomerization state of prokaryotic and eukaryotic glutamate transporters. **a**, Transmembrane segments 2, 4 and 5 form a trimerization domain and these three segments are red, blue and green in each of the three subunits, viewed from the cytoplasm. The yellow and orange spheres indicate the sulphur atoms in a model of the Ser 179 and Asp 185 double cysteine mutant. **b**, SDS–PAGE analysis of the Glt_{ph}7H S179C/D185C mutant, untreated and treated with copper phenanthroline. **c**, Western blot of hEAAT2–GFP cross-linked with glutaraldehyde. Bands I, II and III correspond to monomer, dimer and trimer.

temperature for functional activity. Nevertheless, the presence of this prominent electron density feature, combined with its provocative location, is suggestive of a substrate-binding site in Glt_{ph}H7.

The location of the substrate-binding site is noteworthy because the amino acids that surround the site are conserved across transporter homologues and are critical to functional activity

(Figs 1 and 5). The binding site, of which there is one per subunit, is located below the basin, and is covered by the tip of HP2. In eukaryotic transporters HP2 contains residues that are important for sodium binding. In particular, Ser 440 and Ser 443 in GLT-1, which are equivalent to Gly 354 and Gly 357 in Glt_{ph}H7, are important for sodium selectivity of the transporter³¹. Gly 354 and Gly 357 in Glt_{ph}H7 flank the tip of HP2 and are within 5 Å of the substrate-binding site.

Bounding the other sides of the binding site are the conserved serine residues at the tip of HP1, the β -bridge of TM7, and a polar portion of TM8 (Fig. 5). In TM7 the 'NMDGT' motif contributes to the substrate-binding pocket: the side chains of Met 311 and Thr 314 point towards the binding pocket while Asn 310 and Asp 312 point away from the binding pocket, interacting with each other and with residues in TM3, TM6 and TM8. We suggest that the interactions of Asn 310 and Asp 312 stabilize the β -bridge structure and the binding pocket. Emphasizing the importance of the NMDGT motif, previous studies have shown that conservative point mutants in this region are non-functional¹⁴. In the Glt_{ph}H7 structure, the conserved residues Asp 394, Arg 397, Thr 398 and Asn 401 are on the polar face of the amphipathic TM8 and are positioned to form numerous interactions with the substrate-binding site (Fig. 5; see also Supplementary Fig. S2).

In eukaryotic glutamate transporters the arginine equivalent to 397 in Glt_{ph}H7 confers specificity to substrates with β - and γ -carboxy groups, and mutation of the arginine to a neutral residue results in a transporter that preferentially transports neutral amino acids and that no longer counter-transport potassium³² (Fig. 1; see also Supplementary S2). Two residues implicated in potassium binding and counter-transport in eukaryotic transporters are in contact with or close to Arg 397. The first is Tyr 317 (ref. 33), a conserved residue in TM7, which is involved in a π -cation interaction with Arg 397. The second residue is Gln 318, near Arg 397, which in eukaryotic transporters is a glutamate residue crucial to potassium coupling³⁴. In the Glt_{ph}H7 structure we see that Arg 397 is poised to interact with the γ -carboxy group of glutamate. Even though we do not know precisely how Tyr 317 and Gln 318 couple ion binding to substrate transport, the Glt_{ph}H7 structure demonstrates that residues involved in substrate and ion binding are close in space.

Mechanism

The alternating access mechanism³⁵ is a simple model by which to understand the activity of glutamate transporters. In this model an intramembraneous substrate-binding site is flanked by two gates that allow alternating access of the substrate to either the extracellular or intracellular solution. Here, we suggest the locations and structural features of the gates, substrate-binding site and transport pathway in the Glt_{ph}H7 protein (Fig. 6).

Perhaps the most striking feature of the Glt_{ph}H7 structure is the aqueous basin that allows for substrates and ions to access binding sites approximately halfway across the membrane, directly from bulk solution. Substrate-binding sites are located ~ 5 Å beneath the basin bottom and are secured underneath the tips of HP2. We suggest that HP2 comprises the extracellular gate. Directly under the binding pocket are HP1, TM7a and the C-terminal part of TM8, and we speculate that HP1 forms the intracellular gate because movement of HP1 relative to TM7 and TM8 would open an aqueous pathway from the substrate-binding site to the cytoplasm. Accordingly, in the *Bacillus stearothermophilus* glutamate transporter, residues that map to one face of TM8 and the serine-rich region of HP1 are accessible from intracellular solution^{18,28}. Moreover, in the Glt_{ph}H7 structure there are small cavities along the HP1 and TM8 interface, suggesting that changes in the packing of the helices are plausible.

We propose that the Glt_{ph}H7 structure represents a bound state of the transporter with both gates closed. However, without

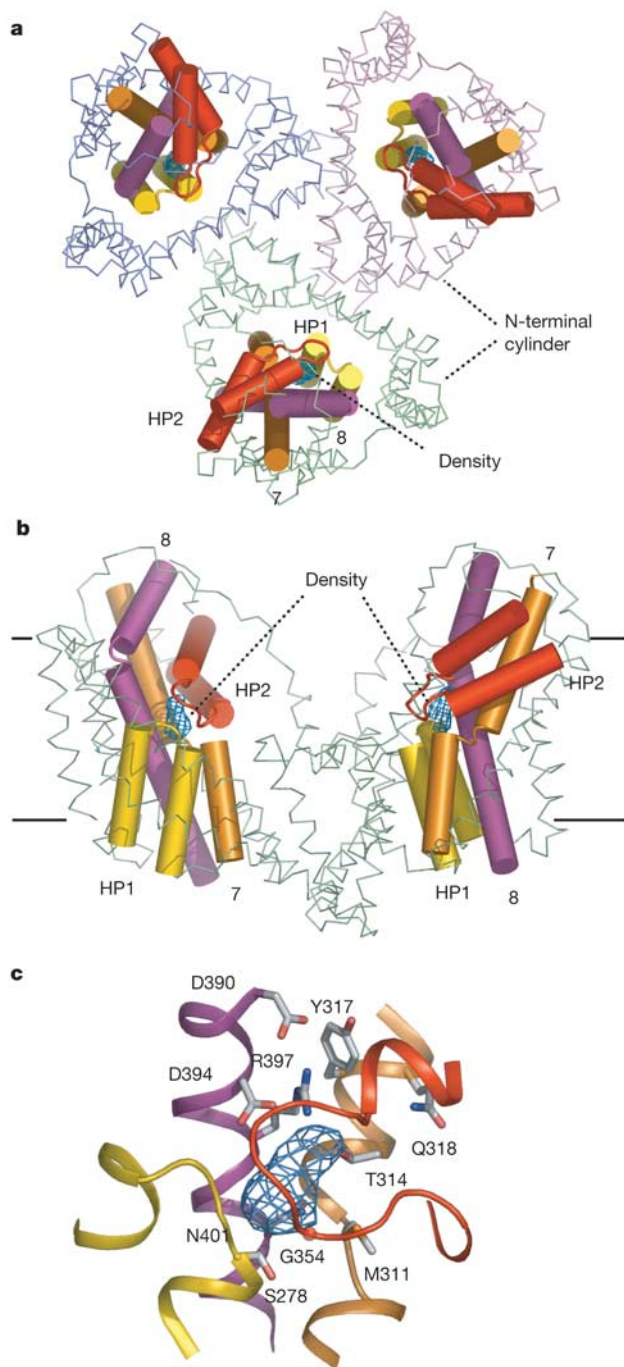


Figure 5 Substrate-binding site is located between the tips of HP1 and HP2. **a, b**, Shown are **a**, Glt_{ph}H7 trimer viewed from the extracellular space and **b**, two subunits viewed parallel to the membrane plane with N-terminal cylinders represented by an α -carbon trace and with HP1, TM7, HP2 and TM8 drawn as cylinders and coloured according to Fig. 3. At the tips of HP1 and HP2 is the non-protein electron density (blue mesh) that defines the substrate-binding site, from a three-fold averaged, $F_o - F_c$ map contoured at 4σ . **c**, A close-up view of the substrate-binding site, with residues implicated in glutamate and ion binding shown in stick representation, together with the non-protein electron density, contoured and coloured as in **a** and **b**.

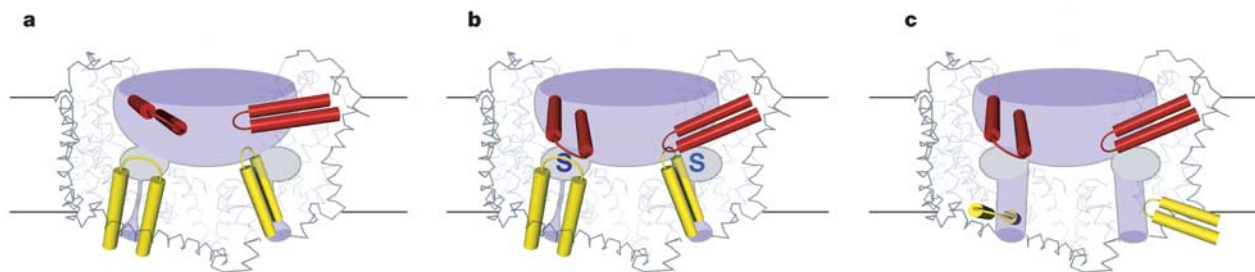


Figure 6 Trimer architecture and mechanism of transport. Glutamate transporters have a large aqueous basin at the bottom of which are located three substrate-binding sites. Here, two of the three substrate-binding sites and transport pathways are shown. Access to the substrate-binding site (shown in grey), from extracellular or intracellular solution, is mediated by HP2 (red) or HP1 (yellow), respectively. **a**, HP2 is in an 'open' conformation, providing access to the binding site from the extracellular basin. **b**, Bound state of the

transporter observed in the Glt_{ph}H7 structure, where access to the binding site is blocked by HP1 and HP2. The substrate and co-transported ions are represented by the letter S. **c**, Movement of HP1 out of the protomer core, towards the cytoplasm and away from the three-fold axis, opens a transport pathway from the substrate-binding site to the cytoplasm.

structures of specific functional states, we can only speculate on the conformational transitions that occur during transport. Nevertheless, biochemical experiments suggest that HP2 undergoes substrate-dependent conformational changes. For example, the solvent accessibilities of residues in HP2 and TM7 are modulated by glutamate and sodium in eukaryotic transporters^{36,37}. Furthermore, fluorescence experiments on hEAAT3 demonstrate that the loop connecting HP2 to TM8 undergoes changes in environment upon glutamate and sodium binding³⁸. We therefore suggest that opening of the extracellular gate involves movement of the HP2 'flipper', perhaps allowing HP2 to pack against and stabilize the TM3–TM4 loop. Consistently, protease sensitivity of the TM3–TM4 loop in GLT-1 is increased in the presence of sodium and glutamate²⁷.

Even though HP1 and HP2 harbour a marked structural similarity they are located in different protein contexts and therefore the conformational changes they undergo during gating, as well as the chemical cues that activate gating, are probably distinct. To open the intracellular gate we suggest that HP1 moves vertically towards the cytoplasm and laterally into crevice 2 (Figs 2b and 6c), thereby creating a substrate transport pathway along the polar face of TM8 and rendering the serine-rich region of HP1 accessible to the cytoplasm.

When the intracellular gate is open we suggest that HP2 moves towards the centre of the trimer, occupying the space vacated by the tip of HP1, thereby preventing the formation of an open transmembrane pore. Indeed, movement of HP2 is consistent with the observation that in human EAAT1 a cysteine introduced into HP2 forms a disulphide bond with a cysteine in TM2 (ref. 13); the equivalent residues in Glt_{ph}H7 are separated by ~20 Å. Indeed, chemical modification of cysteines on the surface of HP2 arrests transport but not substrate binding^{39–41}, suggesting that HP2 may participate in packing interactions different from those observed in the crystal structure. Finally, the intracellular accessibility of the Ala 432 to Cys mutant in GLT-1, which provided the basis for a proposed second re-entrant loop in glutamate transporters¹⁵, is inconsistent with the position of HP2 in the Glt_{ph}H7 structure because the equivalent residue, Ala 345, is located in the middle of HP2a and not at the tip of HP2. We suggest that movement of HP2 towards the substrate-binding site could expose Ala 345 to the intracellular solution and 'seal' the transport pathway.

Discussion

The architecture of glutamate transporters is well suited for the rapid binding of glutamate in synapses. The large aqueous basin allows transmitter to diffuse, through bulk solution, to readily accessible binding sites halfway across the membrane bilayer. Once bound, rearrangements of the cytoplasmic HP1, and perhaps additional elements of structure, open a pathway through each

subunit to the cytoplasm. Although the Glt_{ph}H7 structure defines the gates that allow alternating access of the binding site to either side of the membrane, many important questions remain unanswered, including the location of ion binding sites, the molecular mechanism coupling ion and substrate binding, the location of the chloride permeation pathway and, most importantly, the conformational changes that accompany each step in the transport cycle. □

Methods

Protein preparation

Unlabelled Glt_{ph} and Glt_{ph}H7 were expressed as His₈ fusion proteins, using the pBAD24 vector and *Escherichia coli* Top10 cells⁴², and both proteins were expressed and purified as described previously²². Purified protein was dialysed against a crystallization buffer containing (in mM): 10 HEPES, 25 NaCl, 25 KCl, 1 EDTA, 5 L-glutamate and 7 β-decyl maltoside. Selenomethionine-substituted proteins were expressed in LMG194 cells, and purified in the presence of 2 mM β-mercaptoethanol. Selenium incorporation, as determined by mass spectrometry, was >95%.

Crystallization

Hexagonal rod crystals were grown by vapour diffusion at 4 °C by mixing equal volumes of protein (7–10 mg ml⁻¹) and reservoir solution containing 14–18% PEG 1000, 100 mM Li₂SO₄, 50 mM citric acid, 50 mM Na₂HPO₄. Prior to flash-cooling in liquid nitrogen, crystals were cryo-protected using a reservoir solution adjusted to 30% PEG 1000 with 5% glycerol. The platinum derivative was prepared by soaking crystals in a solution containing 50 mM K₂Pt(NO₃)₄ for 6 h followed by a 1 h back-soak.

Structure determination

Diffraction data sets were indexed, integrated and scaled using HKL2000 (ref. 43) and CCP4 programs. For the Pt MAD data set, initial heavy atom sites were found using Solve⁴⁴ and were refined with SHARP⁴⁵. MAD phases to 8 Å were applied to the native data set and gradually extended to 3.5 Å using the three-fold averaging, solvent flattening and histogram matching in DM⁴⁶. An initial model was built using the program O⁴⁷ and refinement was carried out using REFMAC²³ and CNS⁴⁸ with tight three-fold NCS restraints. To determine selenium atom positions in selenomethionine derivatives, anomalous difference maps were calculated using density-modified phases. Because the V231M mutant was non-isomorphous, the initial phases were obtained by molecular replacement using AMoRe⁴⁹.

Cross-linking

The Glt_{ph}H7 double mutant, S179C/D185C, was expressed and purified as described above, and either left untreated or treated with 1.5 μM of Cu(II) (1,10-phenanthroline)₃. The samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE), and by mass spectrometry, under non-reducing conditions.

From a stable HEK 293 cell line expressing hEAAT2–GFP–His₈ we determined that the fusion construct was active in ³H-glutamate uptake using standard procedures¹⁴. For cross-linking experiments, membranes were solubilized using β-dodecyl maltoside and the transporter was partially purified by size exclusion chromatography. The GFP fluorescent peak was subsequently cross-linked with glutaraldehyde at 25 °C for 30 min and the reactions were quenched using 150 mM Tris-HCl, pH 7.5. The extent of cross-linking was evaluated by western blotting using an anti-His antibody.

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Correspondence and requests for materials should be addressed to E.G. (jeg52@columbia.edu). The coordinates for the structure are deposited in the Protein Data Bank under accession code 1XFH.

A fossil origin for the magnetic field in A stars and white dwarfs

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Some main-sequence stars of spectral type A are observed to have a strong (0.03–3 tesla), static, large-scale magnetic field, of a chiefly dipolar shape: they are known as ‘Ap stars’^{1–4}, such as Alioth, the fifth star in the Big Dipper. Following the discovery of these fields, it was proposed that they are remnants of the star’s formation, a ‘fossil’ field^{5,6}. An alternative suggestion is that they could be generated by a dynamo process in the star’s convective core⁷. The dynamo hypothesis, however, has difficulty explaining high field strengths and the observed lack of a correlation with rotation. The weakness of the fossil-field theory has been the absence of field configurations stable enough to survive in a star over its lifetime. Here we report numerical simulations that show that stable magnetic field configurations, with properties agreeing with those observed, can develop through evolution from arbitrary, unstable initial fields. The results are applicable equally to Ap stars, magnetic white dwarfs and some highly magnetized neutron stars known as magnetars. This establishes fossil fields as the natural, unifying explanation for the magnetism of all these stars.

For a fossil field to survive over millions of years, it has to be stable on an Alfvén timescale (the time for an Alfvén (magnetic) wave to cross the star, of the order of years for the observed field strengths). In addition there is the unavoidable decay of the field caused by the finite electrical resistivity of the plasma, but for an A star this is a slow process.

Although there have been educated guesses as to what shape a field in a stable equilibrium might have^{8,9}, all configurations studied with the analytic methods available so far were found to be unstable on the Alfvén timescale; it has been impossible to prove stability for even a single field configuration^{10,11}. But where analytic methods fail, numerical simulations can sometimes be useful. The field configurations sought here, if they exist, are global, with length scales of the order of the size of the star. This makes them amenable to full time-dependent three-dimensional numerical simulations. This is in contrast to dynamo-generated magnetic fields like that of the Sun, which have global length scales as well as very small length scales that cannot be simultaneously resolved with current technology.

With a grid-based magnetohydrodynamics code¹², we model a non-rotating, self-gravitating body of electrically conducting plasma with pressure and temperature profiles approximating those of a real star (a polytrope of index $n = 3$). The initial magnetic field is random, with the smallest length scales dominating. The initial ratio of magnetic to thermal energy density is roughly constant through the star. From this state, the field decays quickly at the beginning of the simulation. However, after a few Alfvén timescales, the decay slows down as a stable equilibrium is approached. Remarkably, this equilibrium was found to be always of the same general shape, regardless of the particular realization of the random initial field. It is roughly axisymmetric, consisting of both toroidal (azimuthal) and poloidal (meridional) components, in the shape of a roughly circular torus. Its structure is consistent with guesses based on previous analytical work^{8,10,13} which suggested that any stable field configuration must be twisted, with a mixture of poloidal and toroidal fields of similar strengths.

The orientation of the torus and the handedness of its twist (right- or left-handed) depend on the particular realization of the initial random field. Usually the resulting torus is also slightly distorted and somewhat offset from the centre of the star. An

example of such a field configuration is shown in stereographic view in Fig. 1a. Figure 1c is a cross-section of this field, and Fig. 1d is a schematic representation. The surface field strength also depends on the initial conditions, but stable fields as high as 1 T are easily reached. In the atmosphere, the field is of the poloidal, ‘offset dipole’ type seen in most observations, but the structure below the surface is quite different. This must be so, because any untwisted, purely poloidal field in the star is known to be highly unstable^{10,14,15}. This can be seen by considering a configuration consisting of a pure dipolar field outside the star, matched onto a uniform field inside¹⁶. With respect to a plane, parallel to the field lines, which divides the star into two halves, this configuration is like two bar magnets parallel to each other with their north poles adjacent. The magnets will tend to rotate until the north pole of one is next to the south pole of the other. The same happens to a star with a purely poloidal field: one half of the star can rotate by 180° with respect to the other half. We have numerically followed the evolution of this example, and found the field to decay completely in a few Alfvén timescales. In the configurations starting with random fields, on the other hand, the decay is halted by the azimuthal field component of the twisted torus. It provides stability, as long as it carries enough magnetic flux.

Once this stable field has formed it continues to evolve, albeit on a much longer timescale, as a result of electrical resistivity. Following this evolution numerically, we find that the field strength on the surface of the star now increases again, as a result of gradual outwards diffusion of the field lines, even though the magnetic energy as a whole is decreasing. Scaling the results to realistic values of the resistivity, the timescale of this increase corresponds to around 2×10^9 years. This agrees with recent observations¹⁷ which suggest that the A stars with detectable magnetic fields are about 30% older than normal A stars. In the course of this outward diffusion phase, the shape of the field gradually changes. The potential field in the atmosphere does not support twisted field lines, as this would require the atmosphere to carry an electric current. Field lines of the torus therefore ‘unwind’ when entering the atmosphere. The toroidal component, which initially accounts for most of the magnetic energy, thereby decreases until the poloidal component starts dominating. When this point is reached, the field becomes distorted in just the way expected from the aforementioned bar-magnet analogy (stereographic view in Fig. 1b). After this the evolution speeds up, and the field disappears within a short time.

The existence of the stable equilibria found here can be interpreted in terms of magnetic helicity, a concept which has also been useful in other contexts—for instance, in the magneto-hydrodynamics of the solar corona¹⁸ and in laboratory plasma experiments¹⁹. Defined as the volume integral of the dot product of magnetic field with its vector potential, magnetic helicity is a measure of the net ‘twist’ of a field configuration. It is strictly conserved in the absence of reconnection of field lines, that is, when the resistivity vanishes. A magnetic field of non-zero magnetic helicity residing in a perfectly conducting fluid cannot, therefore, decay to nothing. Instead, it relaxes to a stable equilibrium (a local minimum of magnetic energy) with the same value of magnetic helicity²⁰. In the laboratory, a tendency towards helicity conservation is seen even when resistivity cannot be ignored¹⁹. In our simulations, strict conservation does not hold either (mostly because helicity can leave the star through the atmosphere), but the tendency is still strong enough to allow stable equilibria to form in the interior.

We have modelled non-rotating stars, guided by the observations, which do not show systematic differences between rapidly and slowly rotating stars. Rotation also introduces an additional timescale, making the calculations an order of magnitude more expensive. It is conceivable, however, that rotation in fact has a significant influence on the initial magnetic helicity present, and on observables like the orientation of the magnetic axis in the final state. The effect of the convective core, not modelled here, is expected to be small, as its radius is only around one-tenth of the

radius of the star, and contains only a negligible fraction of the magnetic energy of the configuration. The smallness of the convective core is a problem for the dynamo theory of A-star fields, as it would require an implausibly large field strength in this core to produce surface fields as large as a tesla. A second difficulty for this theory is that the observations show no correlation between rotation (an essential ingredient for a dynamo) and field strength.

The field configurations resulting from our quantitative numerical calculations reproduce the key observations: the static nature of the field on historical timescales, their 'offset dipole' shape, the observation that the presence of a field does not depend on the star's rotation, the large strengths attainable¹⁻⁴, and the observational indication that Ap stars may be somewhat older on average¹⁷. The deviations from a simple dipole are still significant at the end of the fast initial evolution of the field, before magnetic diffusion simplifies the configuration further on a slower timescale. From this we predict that the strongest deviations from a dipole should occur in the youngest stars. The results also predict that the field will disappear in a final phase of rapid evolution, though it is not

quite certain that this will happen within the star's lifetime.

The results presented here also apply to objects other than Ap stars. A subgroup of white dwarfs possess a strong magnetic field of similar geometry and total flux to the Ap stars^{21,22}. Because, unlike the Ap stars, white dwarfs contain no convective core, dynamo processes cannot account for this magnetic field. In addition, there is a small class of neutron stars, the magnetars²³, known for their X-ray outbursts²⁴, which possess a very strong magnetic field, of the order of 10^{11} T (refs 25, 26). In other neutron stars, for instance the classical pulsars, the field is weak enough to be held in place by the solid crust on the surface, whereas the strong field of a magnetar would be able to break the crust. A slowly evolving stable configuration in the fluid interior, of the type found here, would explain both the long-term stability of a magnetar's field and the periodic cracking of the crust observed as X-ray outbursts.

The structure and stability of the magnetic fields in these three types of object can thus be understood by a single process: the spontaneous evolution of a magnetic field under the action of its own dynamics in a self-gravitating fluid. □

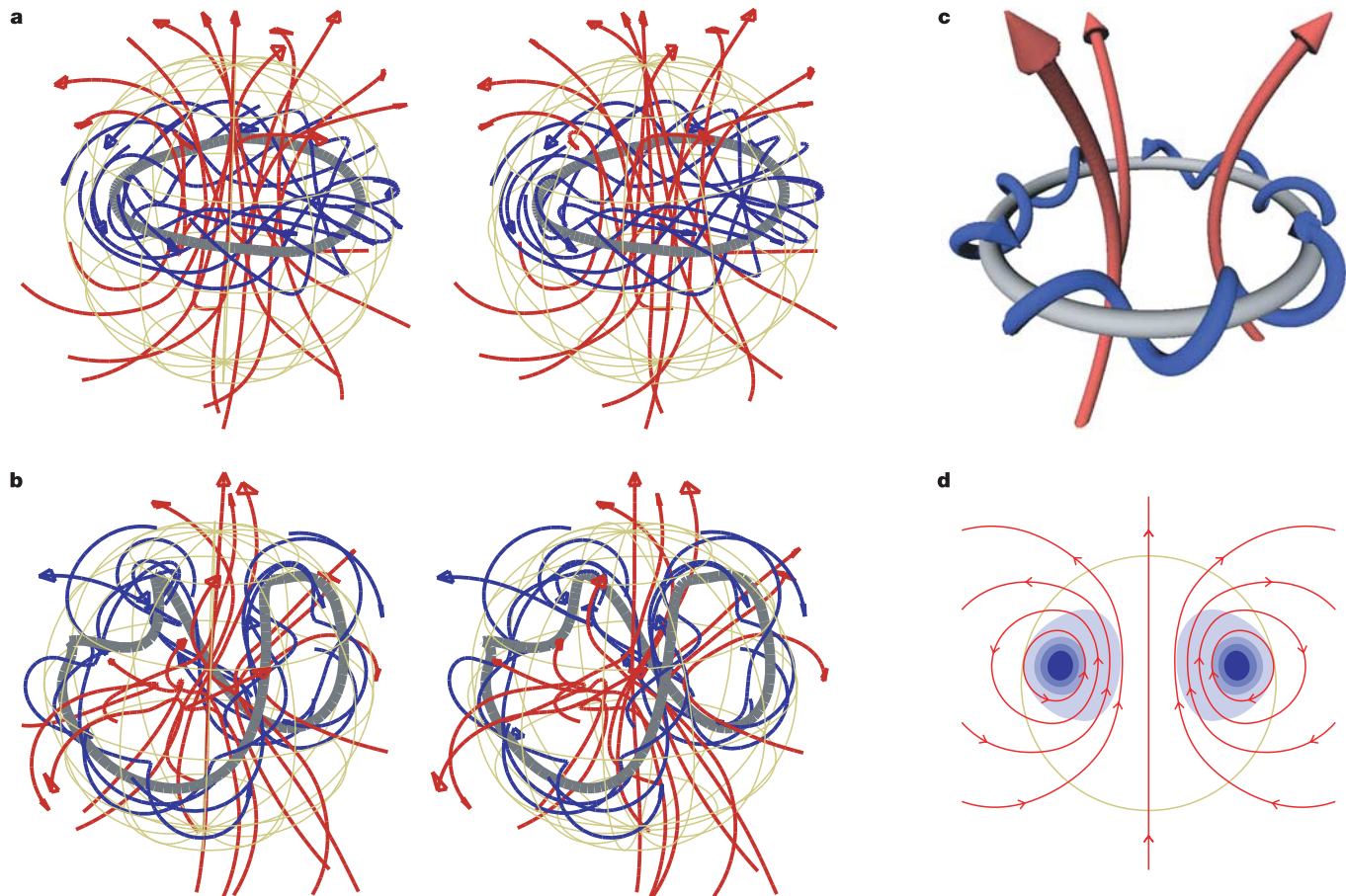


Figure 1 Structure of the stable magnetic fields of A stars and magnetic white dwarfs, as found with three-dimensional numerical simulations. **a**, Stereographic view of the long-lived magnetic field configuration as it evolves from a random initial condition. The stable core of the configuration is formed by a torus of twisted field lines inside the star (blue, with axis of torus shown in grey). Field lines that pass through the stellar surface (red) are stabilized by the torus. The configuration slowly evolves outwards by magnetic diffusion. When the torus reaches the surface it becomes unstable (stereographic view, **b**) and distorts into a shape like the seam on a tennis ball. The initial field configuration for the simulations is constructed from a randomly generated vector potential. The spectrum of spatial wavenumbers k of the variations in the resulting magnetic field is flat (energy spectrum $E(k)$ is a constant). This initial field is tapered such that the ratio of magnetic to thermal energy density in the stellar interior is about 1%, roughly constant as a function of radius. Twenty different random realizations were used. Vacuum conditions outside the

star are modelled by embedding the model star in a tenuous, poorly conducting atmosphere. This guarantees that the magnetic field outside the star's surface stays close to its lowest energy state, a potential field. The numerical code used¹² is a grid based, explicit MHD code, sixth order in spatial dimensions and third order in time (predictor-corrector routine). It was run at a resolution of 144^3 during the initial evolution of the field on the dynamical (Alfvén) timescale, and 96^3 to model the slower, diffusive evolution of the stable field and its eventual decay. The value of the electrical resistivity was set such that the magnetic diffusion time was 100 times the initial Alfvén timescale. **c**, An azimuthal average of the toroidal (shaded in blue) and poloidal (red lines) field components.

d, Diagram showing the axis of the torus field (grey), the torus field lines which are closed within the star (blue) and the untwisted, poloidal field which emerges into the atmosphere (red). The surface of the star, as in **a** and **b**, is shown in yellow.

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Jarosite as an indicator of water-limited chemical weathering on Mars

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The Mars Exploration Rover Opportunity identified the ferric sulphate mineral jarosite and possible relicts of gypsum at the Meridiani Planum landing site¹. On Earth, jarosite has been found to form in acid mine drainage environments, during the oxidation of sulphide minerals², and during alteration of volcanic rocks by acidic, sulphur-rich fluids near volcanic vents³. Jarosite formation is thus thought to require a wet, oxidizing and acidic environment. But jarosite on Earth only persists over geologically relevant time periods in arid environments because it

rapidly decomposes to produce ferric oxyhydroxides in more humid climates⁴. Here we present equilibrium thermodynamic reaction-path simulations that constrain the range of possible conditions under which such aqueous alteration phases are likely to have formed on Mars. These calculations simulate the chemical weathering of basalt at relevant martian conditions. We conclude that the presence of jarosite combined with residual basalt at Meridiani Planum indicates that the alteration process did not proceed to completion, and that following jarosite formation, arid conditions must have prevailed.

The occurrence of sulphate alteration phases in martian meteorites^{5,6} and previous observations of significant concentrations of sulphur at the Viking⁷ and Pathfinder⁸ landing sites support the recent Mars Exploration Rover (MER) evidence that sulphur has played an important role in Mars' surface geochemical processes. Atmospheric sulphur released by volcanic outgassing^{9,10} appears to have been incorporated into the regolith through aqueous alteration or solid–gas interactions to produce sulphur-bearing phases^{11–14}. Analyses of Viking and Pathfinder Lander data indicate that sulphur observed in Mars' regolith is probably associated with magnesium sulphate salts^{6,15}. However, early thermodynamic simulations predicted iron sulphates to be the most likely phases to form at Mars' surface conditions. It should be noted that in this previous model iron sulphates were thought to form from solid–gas alteration of troilite and pyrrhotite, not through aqueous alteration processes¹⁶. Two models have been presented to account for the salts observed in the surface materials. The first assumes that early Mars was warm and wet, creating salty oceans that later evaporated, leaving behind evaporite minerals¹⁷. The second suggests that the sulphur phases formed relatively recently as a result of 'acid fog' reacting with surface materials to form secondary salts¹¹.

Equilibrium thermodynamic reaction-path simulations that constrain the range of possible conditions under which aqueous alteration phases are likely to form are presented here. Using the numerical thermodynamic reaction-path model REACT¹⁸ we have simulated chemical weathering of basalt at Mars-relevant conditions. The results provide a basis for interpreting the geochemical history of acidic chemical weathering on Mars. In these equilibrium models, a given quantity of a basaltic mineral assemblage¹⁹ consisting of diopside, enstatite, ferrosilite, K-feldspar, anorthite, albite, fayalite, forsterite and magnetite is titrated into 1 litre of aqueous fluid containing variable concentrations of sulphate and trace amounts of Na⁺, K⁺, Ca²⁺, Fe²⁺, Mg²⁺, Al³⁺ and dissolved SiO₂. The model includes kinetics only through the suppression of mineral phases unlikely to form in a geologically relevant time period. Suppression of mineral phases is done at the discretion of the operator based on the feasibility of such minerals forming as initial chemical weathering products under wet, oxidizing, low-temperature conditions, as recommended by the author of the modelling package¹⁸. Phases suppressed in the models include epidote, phlogopite, muscovite, K-feldspar, andradite, annite, minnesotaite, greenalite, phengite, tremolite, haematite, goethite and nontronite. In acid mine drainage environments, goethite is the thermodynamically stable phase but it is rarely observed. Rather, the metastable phases jarosite and ferric hydroxides occur in this environment because goethite (and haematite) formation rates are very slow^{4,18}.

The model system is charge-balanced with H⁺, and is buffered by current martian atmospheric oxygen and carbon dioxide fugacities²⁰ at 298 K and 10⁴ Pa total atmospheric pressure. Although CO₂ fugacity may have been greater early in Mars' geologic history, increasing CO₂ fugacity has little effect on jarosite stability. However, photolysis of CO₂ to form O₂ would have resulted in higher atmospheric oxygen fugacity than at present, expanding the pH range over which jarosite would form. The temperature used in the models (298 K) is higher than current martian surface conditions (average 220 K). If the weathering fluids at Meridiani Planum were

approximated by the system $\text{H}_2\text{O}-\text{FeSO}_4$, liquid water could have been stable to temperatures as low as 271.3 K, or as low as 211 K if the fluid were an aqueous sulphuric acid solution²¹. The REACT model is only valid along the liquid–vapour curve for water, from 273 K to 573 K. A temperature of 298 K was used in the models because the thermodynamic data are more complete at this temperature. A few models were run at 273 K for comparison, with no obvious differences from those run at 298 K. The difference in pressure between the model conditions and current atmospheric pressure on Mars (~600 Pa; ref. 22) is likely to have little effect on the thermodynamic properties of the aqueous–solid system²³. Some phases observed on the martian surface today (such as anhydrite and haematite) were probably produced by later dehydration of salts and clays. By varying the fluid composition and water:rock mass ratio, we have constrained the range of conditions at which ferric sulphates are likely to form on Mars.

Alteration assemblages containing ferric sulphate (a Na-end member jarosite phase and/or K-end member jarosite) always include a SiO_2 phase (probably amorphous or microcrystalline when precipitated) and an amorphous iron hydroxide phase (Fig. 1). The minimum concentration of sulphate in aqueous solution required to produce a ferric sulphate phase is of the order of 10^{-5} molal, and the oxygen fugacity of the system must be greater than 10^{-50} . Sodium concentrations $>10^{-7}$ molal are required to produce Na-jarosite (the ferric sulphate phase predicted to precipitate in greatest abundance), while potassium concentrations must exceed 10^{-11} molal to precipitate K-jarosite. The ubiquitous presence of SiO_2 in the alteration assemblages suggests that the widespread high-silica signature observed in Mars' surface thermal emission spectra²⁴ represents secondary silica coatings on surface materials.

Under most conditions jarosite is also accompanied by kaolinite (although kinetics may favour the formation of its polymorph halloysite²⁵ or amorphous allophane²⁶) and gypsum. For gypsum to form, calcium concentrations greater than 10^{-3} molal are required. Subsequent lower humidity levels would probably dehydrate gypsum to form anhydrite, facilitating physical weathering of alteration rinds. This dehydration process may also alter jarosite to less hydrous ferric sulphates and alter iron hydroxides to haematite. Dolomite and calcite are also predicted to form in most models, while dawsonite and the zeolite clinoptilolite are also predicted to form in some cases when initial sulphate concentrations are varied.

The water:rock ratio does not affect the weathering path through E_h –pH space (bold line in Fig. 2), but reaction progress along this path is controlled by the amount of water available to react with the rock (symbols on bold line in Fig. 2). The initial sulphate-rich fluid has a pH of 1. However, the fluid pH increases as the basalt is weathered. This results in the precipitation of jarosite and gypsum

early in the weathering process. Jarosite becomes unstable and is replaced by other Fe-bearing phases in the alteration assemblage as weathering proceeds and pH increases (Figs 1 and 2). The final pH of the system (as defined by that point at which the fluid is in equilibrium with the mineral assemblage present) increases with decreasing water:rock ratio, thus moving the extent of the reaction farther along the weathering path into the iron hydroxide + gypsum field. As a result, the presence of jarosite within alteration assemblages can be used as an indicator of the extent of chemical weathering at Mars' surface conditions. It is only possible for jarosite to form and remain stable if the alteration process is arrested after only a few per cent of basalt weathering occurs.

As the water:rock ratio is decreased, jarosite is formed earlier in the chemical weathering process (>80% of basalt must weather to produce jarosite at water:rock = 100:1, while <20%, <2% and <0.2% of basalt must be weathered at water:rock = 10:1, 1:1 and 1:10, respectively). This suggests that jarosite may form in two ways: (1) from the complete reaction of a large quantity of water with a small amount of rock, that is, a large amount of water altering only a thin outer layer of rock; or (2) from a small amount of water partially weathering a large quantity of rock. In both cases, water must be removed from the system (probably via evaporation) in order to arrest the alteration process before the weathering fluid pH increases and is no longer in the jarosite stability field. This in turn suggests that the weathering rind formed in a geologically short period of time. However, the presence of gypsum without accompanying jarosite would indicate that chemical weathering was extensive, allowing sufficient interaction with the basalt to raise the pH of the weathering fluid beyond the jarosite stability field.

Thermodynamic simulations indicate that reaction of acidic aqueous fluids with basalt under oxidizing conditions produces jarosite and gypsum. However, for jarosite to survive in the

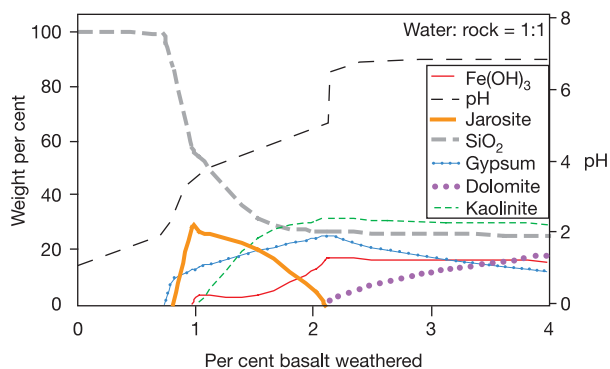


Figure 1 Predicted alteration minerals, reported as weight per cent of alteration assemblage at a water:rock ratio of 1:1. Note that jarosite is only stable during the very earliest stages of basalt weathering.

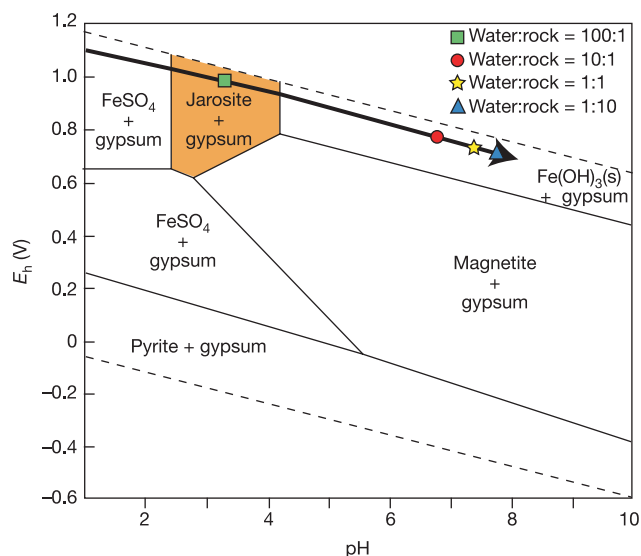


Figure 2 E_h –pH phase diagram of the Fe–S–Ca–Na– HCO_3 – H_2O system at 298 K. Activities of SO_4^{2-} , Ca^{2+} and Na^+ are set at 10^{-2} , 10^{-2} and 10^{-3} , respectively. The dashed parallel lines represent the upper and lower bounds on water stability. The bold line at the upper left of the diagram shows the reaction path followed during weathering of basalt by sulphate-bearing aqueous fluids, buffered by the present martian atmosphere. Symbols on this line represent the extent of the reaction corresponding to different water:rock ratios, as shown in the key at upper right. As the reaction path moves to higher pH, it passes through the jarosite + gypsum stability field. However, if pH continues to rise with increased weathering of the basalt, the reaction path moves out of the jarosite stability field, resulting in dissolution of jarosite and reprecipitation as other ferric iron-bearing minerals in the alteration assemblage. Therefore, observations of jarosite would suggest a short-lived period of water-limited aqueous alteration.

weathering assemblage, alteration must have ceased well before the basalt had been significantly weathered (Fig. 1) and subsequent conditions must have remained dry. This suggests that liquid water was only active at the Opportunity landing site in Meridiani Planum for a geologically short period of time. Thus, while other studies indicate that liquid water was present for a relatively long period of time early in Mars' history¹⁷, its distribution must have been spatially variable. Alternatively, the jarosite observed at Meridiani Planum may have formed later in Mars' history during periods of transient liquid water stability; such conditions could have resulted from extremes in orbital obliquity²⁷ and/or large additions of volatiles to the atmosphere from volcanic eruptions²⁸ or bolide impacts²⁹. In any case, the occurrence of residual (unweathered) basalt along with jarosite requires that liquid water was only present for a limited period of time at this location.

On the basis of thermodynamic simulations, one possible chemical weathering scenario for the Meridiani Planum site is that, as weathering proceeded, the weathering fluids were incorporated into hydrated phases (that is, the rocks literally absorbed the water to form hydrous phases) while simultaneously evaporating. The evaporated water would have over time been lost from the atmosphere by photolytic decomposition, reprecipitated as ice at the poles, or consumed by further weathering rind formation in subsequent periods of aqueous alteration. The lack of liquid water eventually 'stalled' the chemical weathering process early in its history, leaving jarosite or other ferric sulphates as stable alteration phases within the partially weathered basalt. Continued water loss from the planet's atmosphere by photolytic decomposition gradually reduced the relative humidity to levels where hydrous phases exposed to the atmosphere could dehydrate, producing haematite and anhydrite from iron hydroxides and gypsum. Subsequent physical erosion and transport of such alteration rinds may have produced the globally distributed salty fines enriched in sulphur that have been observed at the Viking and Pathfinder field sites³⁰. □

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No enhancement of fusion probability by the neutron halo of ⁶He

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Quantum tunnelling through a potential barrier (such as occurs in nuclear fusion) is very sensitive to the detailed structure of the system and its intrinsic degrees of freedom^{1,2}. A strong increase of the fusion probability has been observed for heavy deformed nuclei³. In light exotic nuclei such as ⁶He, ¹¹Li and ¹¹Be (termed 'halo' nuclei⁴), the neutron matter extends much further than the usual nuclear interaction scale. However, understanding the effect of the neutron halo on fusion has been controversial—it could induce a large enhancement of fusion⁵, but alternatively the weak binding energy of the nuclei could inhibit the process⁶. Other reaction channels known as direct processes (usually

negligible for ordinary nuclei) are also important: for example, a fragment of the halo nucleus could transfer to the target nucleus through a diminished potential barrier. Here we study the reactions of the halo nucleus ${}^6\text{He}$ with a ${}^{238}\text{U}$ target, at energies near the fusion barrier. Most of these reactions lead to fission of the system, which we use as an experimental signature to identify the contribution of the fusion and transfer channels to the total cross-section. At energies below the fusion barrier, we find no evidence for a substantial enhancement of fusion. Rather, the (large) fission yield is due to a two-neutron transfer reaction, with other direct processes possibly also involved.

The development of beams of accelerated unstable ions has offered the possibility of discovering remarkable features in some light exotic nuclei. Effects observed in selected reactions on various target nuclei (dating from the mid 1980s) provided the evidence for an extended spatial distribution of neutron matter, which develops into a 'halo', in systems such as ${}^6\text{He}$, ${}^{11}\text{Li}$ or ${}^{11}\text{Be}$ (refs 7, 8). Also, many experimental results required the abandonment of the picture where the nucleons are bound in a mean field of the nuclear force, and the adoption of a description in terms of interacting clusters of nucleons⁹. From the beginning, much attention has been paid to the effects that these properties might have on fusion probabilities. The neutron halo could help the attractive nuclear forces to begin acting at a larger distance, effectively lowering the barrier. Properties such as deformation, low-lying resonances in the continuum, and strong reaction channels may act as additional degrees of freedom and cause enhancement of the fusion cross-section, as earlier observed in heavier systems^{10–12}. In particular, the small binding energy increases the importance of the breakup channel (dissociation of the projectile in the field of the target); however, its effect on fusion is strongly disputed. If considered as any other channel, it should enhance the sub-barrier cross-section^{5,13,14}; on the other hand, the breakup process could just prevent the capture of the whole projectile, thereby inhibiting fusion^{6,15–17}. Here we use 'fusion' in the sense of complete fusion^{14,17–19} (the whole projectile and target fuse together; evaporation of fragments may follow) as distinct from incomplete fusion (only a fragment of the projectile is transferred to the target; in light nuclei this is not distinguishable from a direct transfer).

The halo-nucleus ${}^6\text{He}$ presents the peculiar properties mentioned above, and has a relatively small breakup threshold (the two-neutron separation energy is $S_{2n} = 0.973$ MeV). It is a favourable candidate for experimental studies, because it is available as a beam of accelerated ions at various facilities. However, the weak intensity

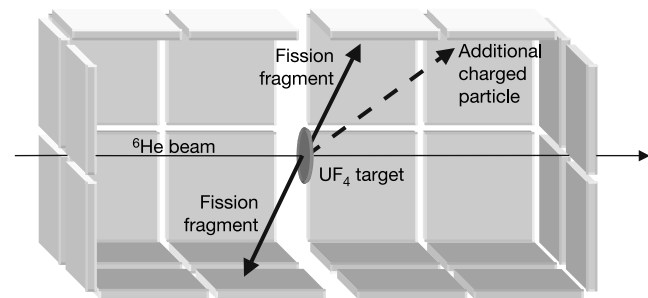


Figure 1 Schematic view of the experimental configuration. The ${}^6\text{He}$ beam impinges on the $500\text{ }\mu\text{g cm}^{-2}$ UF_4 target, which is surrounded by an array of forty $50 \times 50\text{ mm}^2$ silicon detectors (some detectors are omitted in the drawing) covering about 70% of the total solid angle. For each particle hitting a detector, its energy and time of flight (with respect to the cyclotron radio frequency) were recorded. Fission fragments are emitted back-to-back and identified as they hit opposite detectors. Occasionally a direct reaction may cause fission: such events are characterized by the emission of a third charged particle in coincidence. The arrangement of the detectors allows integrated yields in six angular ranges to be obtained.

of these radioactive beams sets a limit on the accuracy attainable in cross-section measurements, and the results obtained so far have not been conclusive. An enhancement was reported in the ${}^6\text{He} + {}^{209}\text{Bi}$ fusion cross-section at energies below the potential barrier²⁰, and then related to the presence of strong breakup and/or transfer channels²¹. Our previous measurement with ${}^6\text{He}$ on ${}^{238}\text{U}$ target nuclei²² also showed a large probability for the sum of all processes leading to the fission of the residual nucleus. The measurement reported here, however, allows the identification of the contribution of incomplete fusion (transfer) channels, which are found to be the most important in the total reaction cross-section.

By employing a fissile ${}^{238}\text{U}$ target, we chose fission as the signature for the events of interest. Complete fusion events produced a highly excited compound system (excitation energies were $E^* \geq 20$ MeV, while the fission barrier $E_{\text{FB}} = (5.4 \pm 0.3)$ MeV, ref. 23), which then decayed, emitting two heavy fragments almost back-to-back. The fragments were detected in coincidence in a highly efficient array of charged-particle detectors, and identified by their energy and relative emission angle. A schematic view of the set-up is given in Fig. 1. The ${}^6\text{He}$ radioactive beam was provided by the two coupled cyclotrons of the Cyclotron Research Centre²⁴ in Louvain-la-Neuve, Belgium. After production in a thick LiF target, using the intense proton beam provided by CYCLONE30, the ${}^6\text{He}$ atoms were extracted, ionized and then injected and post-accelerated in the CYCLONE110 cyclotron, to energies between 15 MeV and 29.5 MeV. The second cyclotron also acts as a mass separator, delivering a beam essentially free of isobaric contaminations. The beam intensity (average 5×10^5 particles s^{-1}) was measured either directly in a plastic scintillator or via the Rutherford scattering on the U nuclei at small angles. The target, produced at the Gesellschaft für Schwerionenforschung laboratory in Darmstadt, Germany, consisted of a UF_4 layer ($500\text{ }\mu\text{g cm}^{-2}$) deposited on a thin C foil ($30\text{ }\mu\text{g cm}^{-2}$). Absolute cross-sections were calculated from the number of detected events, normalizing for beam intensity, target thickness and detection efficiency (measured with a ${}^{252}\text{Cf}$ fission source and an α -particle source).

Measured fission cross-sections are shown in Fig. 2 as functions of the centre-of-mass energy, for both the ${}^6\text{He}$ beam and a beam of

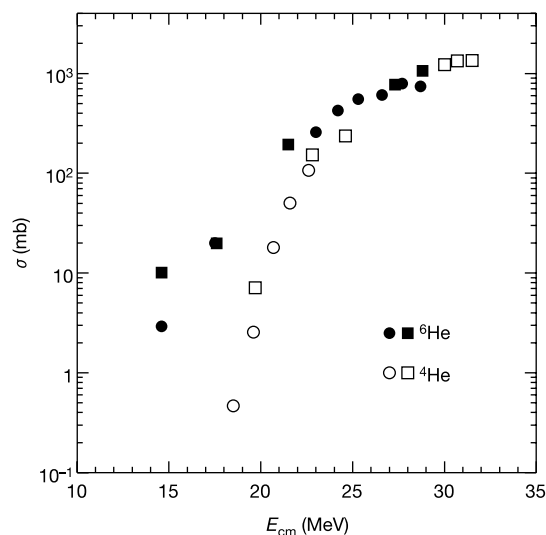


Figure 2 Fission cross-section, σ , for ${}^6\text{He}$ (filled symbols) and ${}^4\text{He}$ (open symbols) on ${}^{238}\text{U}$ as function of the centre-of-mass energy of the reaction, E_{cm} . Data obtained in this measurement (squares) are completed by the ones obtained previously²² (circles). Statistical uncertainties are small and not visible; the overall systematic uncertainty is estimated to be around 15%. Data from ref. 22 have been renormalized by a factor 0.54 after a correction of the set-up efficiency, due to a contamination in the Cf source used for its evaluation.

stable, strongly bound ^4He projectiles. The findings of the previous measurement²² (that is, a large cross-section for ^6He with respect to that for ^4He at energies below the barrier) are essentially confirmed.

Although all fusion events generate fission, other processes can contribute to the cross-section in Fig. 2. Candidates are inelastic scattering, transfer and breakup reactions, leaving the residual nucleus in an excited state above the fission barrier. Large cross-sections for such direct processes at energies near the barrier have been observed, though not disentangled, in the case of ^6He -induced reactions^{21,25}. With the present measurement we separate these contributions from fusion, and identify the specific process. For events other than fusion, a projectile-like particle is also emitted in the reaction. Processes where the entire charge is transferred to the target are strongly endothermic (the energy balance or 'Q-value' is $Q = -6$ MeV for the transfer of an α -particle from a ^6He projectile; much larger for the breakup of the α -particle), and not enough energy would be available for the target residue to fission. Thus any fragment accompanying fission must be charged, and hence will be detected in our setup. Multiplicity-3 events have been observed in a significant quantity only with the ^6He projectile. They have not been observed with the ^4He projectile, showing that for this system only fusion events contribute to the fission cross-section. This also shows that the probability of a random coincidence is negligible, indicating that a physical process is present in the case of the ^6He projectile. For the latter system, spectra of the third charged particle

are shown in Fig. 3a for the various centre-of-mass energies in the entrance channel.

Identification of the reaction mechanism is based on various factors. The fragments must be α -particles, since tritons (the nuclei of ^3H atoms) and protons have a smaller stopping power and, given the thickness of the detectors (500 μm), would escape, depositing only energies lower than observed. Experimental angular distributions, strongly peaked around the grazing angle (thus shifting to backward angles as the beam energy decreases), indicate that the process is of a direct nature. We can calculate the possible energy of the fragment, requiring that the residual nucleus be left in an excited state leading to fission. Energies for the different processes are shown as hatched regions in Fig. 3a. Excitation of the target nucleus (followed or not by ^6He breakup) would result in projectile fragments with lower energy than observed. The one-neutron transfer has a positive ground-state Q-value ($Q = +2.94$ MeV); however, the resulting ^5He nucleus is unbound (by 800 keV) and would dissociate into a neutron and an α -particle. For each fragment, the momentum provided by the dissociation could sum in all possible directions with the centre-of-mass momentum of ^5He , leading to a spread of several MeV in the energies of the detected α -particle; this spread is not observed. For two-neutron transfer, the ground-state Q-value is $Q = +9.76$ MeV: the residual target nuclei are left in a highly excited state and fission, and α -particles emerge with energies corresponding to the observed ones. Figure 3b shows this situation, by plotting the events as function of the excitation energy in the residue ^{240}U .

The curves in Fig. 3b are results of distorted-wave Born approximation (DWBA²⁶) calculations, performed (using the code Fresco²⁷) for the two-neutron transfer process into ^{240}U , for a large range of excitation energies and transferred angular momenta. An effective optical potential, obtained with the continuum-discretized coupled-channel method (CDCC²⁸), using the same parameters as in ref. 29, was employed to account for the effects of the breakup of ^6He . This is (to our knowledge) the first attempt to describe experimental data for different reaction channels, for a light unstable nucleus around the barrier, in a consistent way (the

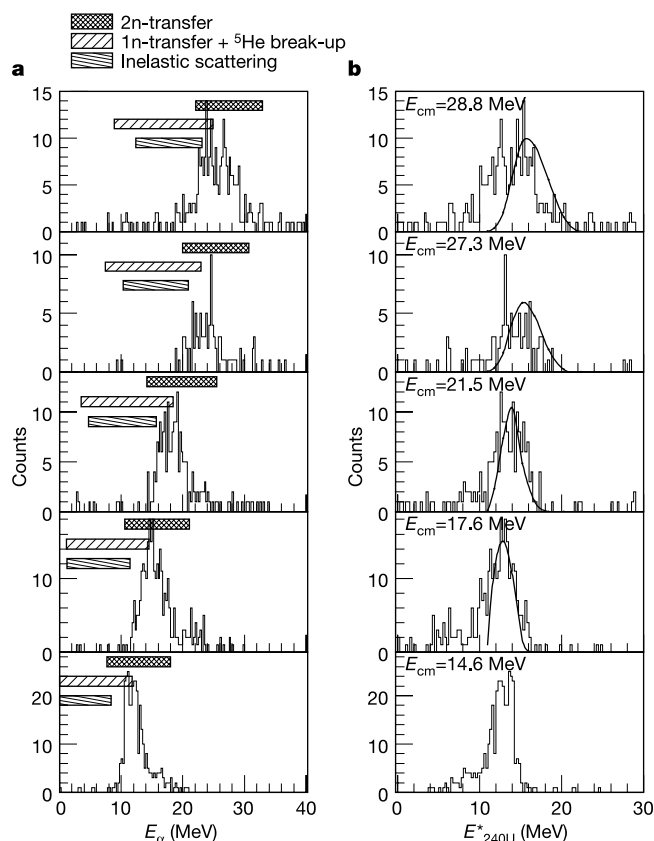


Figure 3 Spectra of charged particles emitted in coincidence with two fission fragments. **a**, Absolute energy of the particles, E_α . The hatched regions correspond to the ranges where particles should appear according to the indicated processes, according to their respective Q-value and requiring that the residual nucleus be left with enough excitation energy to fission. **b**, Same data plotted as function of excitation energy in ^{240}U , $E_{240\text{U}}^*$, to show that the events are two-neutron transfer to excited states in ^{240}U ($E^* \approx 10$ to 15 MeV). The curves are results of DWBA calculations for the two-neutron transfer process into ^{240}U .

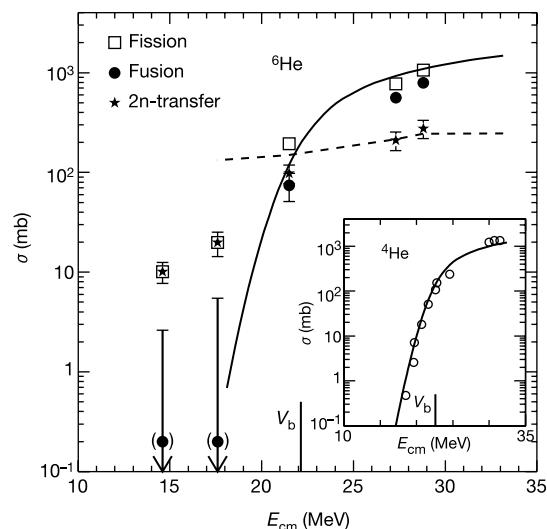


Figure 4 Fusion and two-neutron transfer cross-section for ^6He on ^{238}U (error bars are one standard deviation). Below the Coulomb barrier V_b , there is no enhancement of the fusion cross-section. The curves are results of calculations performed using an effective optical potential, which accounts for the breakup of ^6He . The dotted curve is the two-neutron transfer cross-section in excited states of ^{240}U . The solid curve is the fusion cross-section obtained by employing a short-range imaginary potential to reproduce the incoming wave boundary condition¹². The inset shows the data for ^4He on ^{238}U ; the curve is the fusion cross-section obtained using a short-range imaginary potential.

same effective potential is used to calculate the fusion probability, see below). Approximations had to be introduced: the two neutrons are treated as one cluster, and the model space had to be limited, preventing a calculation at the lowest energy. However, the agreement of the predicted distributions with experimental data (see Fig. 3b) further supports the identification of the transfer process.

The experimental cross-sections for the events in Fig. 3 were calculated as in the case of fission. The detection efficiency depends on the angular distributions, which are not known in the case of the transfer process for such an exotic system. We have used a set of theoretical angular distributions from DWBA calculations in a reduction procedure (fitting the experimental data) to extract the efficiency of each detector, taking into account their geometry. The fusion cross-sections were then calculated as the difference between the total fission and the transfer fission. The resulting data are shown in Fig. 4; the large uncertainty is mainly due to the procedure for the evaluation of the efficiency.

At energies below the barrier, we find experimentally that there is no substantial enhancement of the fusion cross-section for the halo nucleus ${}^6\text{He}$. The large observed yield for fission is entirely due to a direct process, the two-neutron transfer to the target nucleus. Other direct processes may also be present, though not detected owing to the fission signature we required. The coupling to these degrees of freedom, as well as the effect of the halo in ${}^6\text{He}$, is not sufficient to promote an enhancement of the sub-barrier fusion cross-section.

Breakup has been accounted for by using the effective optical potential derived in the CDCC model of the ${}^6\text{He}$ breakup. The fusion cross-section (solid line in Fig. 4) was then obtained by employing a short-range imaginary potential to reproduce the incoming-wave boundary condition¹² (the inset in Fig. 4 shows the same for the ${}^4\text{He} + {}^{238}\text{U}$ system). At the same time, the DWBA calculations provide an estimate of the magnitude of the two-neutron transfer process to excited states in ${}^{240}\text{U}$ (dotted line). Whereas the agreement for the transfer process suffers from the rough approximations used, the fusion cross-section is well reproduced, corroborating our picture for the processes with the halo nucleus ${}^6\text{He}$ at energies around the barrier. Still, the grounds for such picture are primarily in the experimental results presented here: a large reaction cross-section, due to direct processes rather than to an enhancement of fusion. □

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Metal–insulator–semiconductor optoelectronic fibres

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The combination of conductors, semiconductors and insulators with well-defined geometries and at prescribed length scales, while forming intimate interfaces, is essential in most functional electronic and optoelectronic devices. These are typically produced using a variety of elaborate wafer-based processes, which allow for small features, but are restricted to planar geometries and limited coverage area^{1–3}. In contrast, the technique of fibre drawing from a preformed reel or tube is simpler and yields extended lengths of highly uniform fibres with well-controlled geometries and good optical transport characteristics⁴. So far, this technique has been restricted to particular materials^{5–7} and larger features^{8–12}. Here we report on the design, fabrication and characterization of fibres made of conducting, semiconducting and insulating materials in intimate contact and in a variety of geometries. We demonstrate that this approach can be used to construct a tunable fibre photodetector comprising an amorphous semiconductor core contacted by metallic microwires, and surrounded by a cylindrical-shell resonant optical cavity. Such a fibre is sensitive to illumination along its entire length (tens of meters), thus forming a photodetecting element of dimensionality one. We also construct a grid of such fibres that can identify the location of an illumination point. The advantage of this type of photodetector array is that it needs a number of elements

of only order N , in contrast to the conventional order N^2 for detector arrays made of photodetecting elements of dimensionality zero.

Our approach entails the drawing of extended fibre lengths fabricated by arranging a low-melting-temperature crystalline conductor (Sn), amorphous semiconductors (As–Se–Te–Sn and As_2Se_3), and high glass-transition-temperature polymeric insulators (polyetherimide, PEI, and polyethersulphone, PES) into a preform which shares the final fibre geometry but lacks functionality owing to the absence of intimate contact and proper element dimensions. The preform is subsequently heated and drawn into a fibre that preserves the initial geometry while forming intimate interfaces and feature sizes down to below 100 nm. This process has led to the creation of two prototypical functional fibre structures that we discuss in detail here: (1) a fibre designed for dual electron-photon transport, and (2) a tunable, narrow-band fibre photodetector.

The fibre for dual electron-photon transport is comprised of a hollow air core, surrounded by an omnidirectional dielectric mirror^{13–17} formed from eight pairs of alternating layers of As_2Se_3 and PEI (layer thicknesses of 150 and 280 nm, respectively). The refractive indices of As_2Se_3 and PEI are 2.83 and 1.65 respectively at 1.5 μm , as measured by using a Sopra GES5 ultraviolet-visible-infrared spectroscopic ellipsometer (see <http://mit-pbg.mit.edu/Pages/Ellipsometry.html>). This multilayer structure provides the optical confinement to the low-index core. Immediately adjacent to this omnidirectional mirror is a circular array of 60 Sn metal strands

with diameters of $\sim 8 \mu\text{m}$ each (see ‘Preform preparation and fibre drawing’ in Methods). The whole fibre is then surrounded with a PES polymer cladding. An SEM micrograph of the fibre cross-section is shown in Fig. 1a. The optical transport characteristics of the fibre are determined by the positions of the photonic bandgaps. For example, fibres having outer diameters of 980, 1,030 and 1,090 μm have fundamental bandgaps centred at 1.62, 1.75 and 1.85- μm wavelengths, respectively (see Fig. 1c). Optical transmission spectra were determined using a Fourier transform infrared (FTIR) spectrometer (Bruker Tensor 37). The fibre shown in Fig. 1b (1-mm outer diameter) appears green to the eye because of reflection from the third-order bandgap.

We have performed a systematic investigation of the losses incurred upon propagation through simple (non-metal-containing) hollow-core multilayer photonic bandgap fibres in refs 15 and 17. The loss of such fibres for radiation at a wavelength of 10.6 μm was found to be less than 1 dB m^{-1} (ref. 17). More recently, we have characterized such fibres at a wavelength of 1.5 μm , and measured losses to be approximately 5.5 dB m^{-1} (ref. 15). The loss mechanisms are found to be (1) radiation through the multilayers, (2) intrinsic materials absorption, (3) nonuniformity in the layer thicknesses, and (4) scattering due to internal surface roughness. Because the light in the electron-photon fibre is very strongly confined within the hollow core by the cylindrical omnidirectional mirror, it is expected that the presence of additional material components or structural complexity lying outside the cylindrical mirror will not significantly affect the losses through this hollow fibre. Indeed, initial loss measurements on the electron-photon transport fibres yield loss levels that are comparable to the cylindrical photonic bandgap fibres of similar structural characteristics. In particular, broad-band FTIR cut-back measurements on electron-photon fibres of 1 m length and core size 800 μm with eight bilayers yielded a transmission loss value of 4.9 dB m^{-1} , and a bending loss of 0.9 dB for a 90° degree bend with a bending radius of 6 cm. These losses, although higher than those for silica-based holey fibres, are among the lowest reported values for hollow polymer fibres. To examine the electrical properties of the fibre, on the other hand, both ends of the fibre were coated with a thick layer of gold that electrically connects all the Sn wires. The current–voltage characteristics of the 980- μm -thick fibre are displayed in Fig. 1d, showing clear ohmic response.

Whereas the above fibre exhibits both electrical and optical transmission properties, these are spatially separated and indepen-

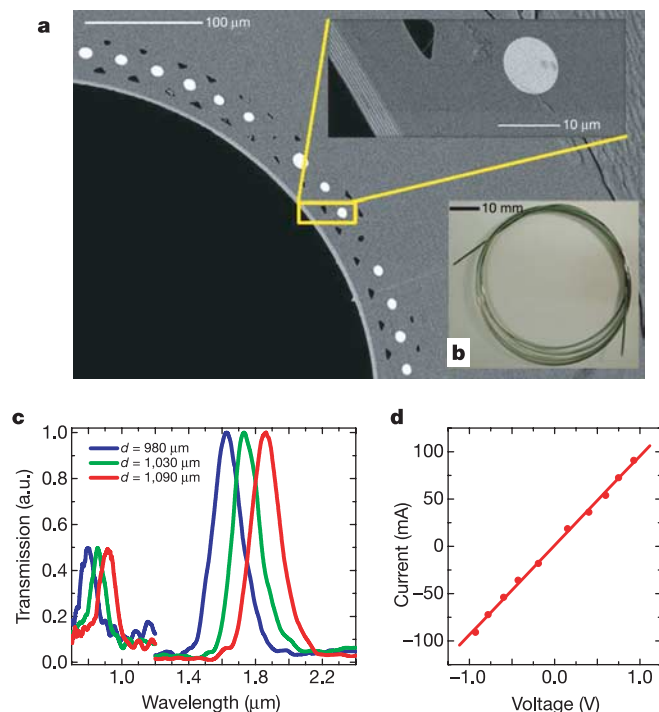


Figure 1 Dual electron-photon fibre (hybrid fibre). **a**, SEM micrograph of the cross-section of the hybrid fibre with 800- μm hollow core, omnidirectional mirror layers, metallic filament array and polymer cladding. The inset shows eight pairs of quarter-wave $\text{As}_2\text{Se}_3/\text{PEI}$ multilayers and one of the metallic, Sn filaments in the ring that is surrounding the mirror layers. **b**, Photograph of a 1-mm-thick, 1-m-long hybrid fibre. The fibre appears green to the eye by virtue of reflection from the third-order photonic band gap of the omnidirectional mirror, located at 550 nm. **c**, Normalized transmission spectra of three different fibres, having outer diameters of 980, 1,030 and 1,090 μm . The primary and second-order photonic bandgaps are located at 1.62 and 0.8 μm for the 980- μm -thick fibre, and are shifted to longer wavelengths as the fibre diameter increases. **d**, Measured electrical current along the 980- μm -thick, 15-cm-long fibre as a function of applied bias voltage.

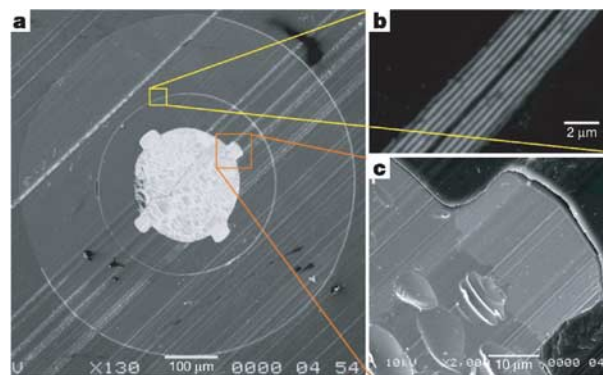


Figure 2 Integrated optoelectronic device fibre. **a**, SEM micrograph of the entire cross-section of a 650- μm -thick device fibre with a 200- μm chalcogenide glass core surrounded by a PES cladding. The core region is surrounded by a resonant cavity structure (eight pairs of $\lambda/4$ $\text{As}_2\text{Se}_3/\text{PEI}$ multilayers, with a $\lambda/2$ resonant cavity in the middle). The bright regions on the polymer–glass interface are the Sn metal electrodes, 18- μm thick and 30- μm wide, which are continuous along the whole fibre length. **b**, A magnified micrograph showing the resonant optical cavity structure. **c**, A magnified micrograph demonstrating the excellent quality of the semiconductor–metal interface.

dent; that is, the propagating electrons and photons do not interact. In our second fibre, we specifically introduce an optoelectronic interaction. This is realized by forming, in the core of the fibre, a photoconductive chalcogenide cylinder contacted with metal electrodes that produces current upon illumination under bias conditions. A single (radial) mode cylindrical-shell optical cavity is introduced in the optical path shielding the photoconducting core from ambient illumination sources. Upon external illumination, when the wavelength of the radiation matches that of the cavity resonance, we observe an electric response from the fibre core, thus establishing the spectroscopic functionality of the fibre.

Figure 2 shows SEM micrographs of the spectroscopic fibre cross-section. The fibre core is $\text{As}_{40}\text{Se}_{50}\text{Te}_{10}\text{Sn}_5$ (AST-Sn), a highly photoconductive glass, optimized subject to the following requirements (see 'Glass synthesis' in Methods): (1) large photoconductivity in the wavelength range of interest; (2) viscosity range compatible with the polymer; and (3) enhanced stability against crystallization during fibre drawing. The core is contacted by four Sn electrodes to form the photodetecting element. An enlargement of the metal–semiconductor interface demonstrating the intimate contact is shown in Fig. 2a. A resonant optical cavity structure is formed by a multilayer made of As_2Se_3 and PEI with a $\lambda/2$ defect layer; an enlargement of the resonant cavity structure is shown in Fig. 2b. The micrograph shown in Fig. 2c demonstrates excellent contact between the semiconducting core and a metal electrode (see 'Preform preparation and fibre drawing' in Methods).

To confirm the fidelity of the metal–semiconductor contact and characterize its photoconductive properties, we measured the broad-band photoconductive response of a two-electrode fibre without a multilayer structure (the red curve in Fig. 3a). A 50-V direct current (d.c.) voltage was applied to the fibre and the current was measured using a pico-ampere meter/d.c. voltage source (Yokogawa/Hewlett Packard 4140B). The fibre is illuminated externally with a laser beam from a tunable Optical Parametric Oscillator (OPO). Optical transmission of bulk glass (12-mm diameter and 5-mm length) was determined by an FTIR measurement (blue curve in Fig. 3a). The spectral photoconductive response is commensurate with the optical transmission measurement.

The resonance wavelength and photonic bandgap are both determined by the outer diameter of the device fibre. We fabricated three fibres of outer diameters 870, 890 and 920 μm , which have calculated resonance wavelengths of 1.26, 1.29 and 1.33 μm , respectively. In these fibres, the multilayer structure was situated at the fibre outer surface. The multilayer structure in the three fibres consists of three bilayers, As_2Se_3 and PEI, a $\lambda/2$ PEI cavity, followed by three more bilayers. For the 920- μm fibre, for example, the thicknesses of As_2Se_3 and PEI layers are 117 and 204 nm, respectively. The reflectivity of the optical cavity structure was measured for single fibres having these diameters with an FTIR spectrometer and microscope (Nicolet/SpectraTech NicPlan infrared microscope and Magna 860 FTIR spectrometer), and are displayed in Fig. 3b. The FTIR spectra agree well with the calculated spectra. The microscope objective admits a range of angles that vary from normal incidence to 35° , so the observed dip in the reflection spectra is wider and shallower. The cavity resonance shifts slightly in wavelength when the angle of incidence changes. Such a large angular cone, as determined by the microscope objective numerical aperture (NA) of 0.58, leads to an averaging effect that reduces the apparent quality factor of the cavity mode.

To characterize the optoelectronic response of our integrated device fibre, it is useful to measure both the electrical photocurrent and the optical reflectivity simultaneously. We externally illuminated the three aforementioned two-electrode device fibres with the OPO laser beam and measured the back-reflected light through a beam splitter, while simultaneously monitoring the generated photocurrent (see 'Optical characterization of device fibre' in Methods).

In Fig. 3c, we display the measurement results of the back-reflected light from three different fibres as wavelength of the laser beam is swept. Concurrently, in Fig. 3d we plot the results of photocurrent measurements of these fibres. At the resonance wavelength, the back-reflection is diminished, and the light reaches the photoconductive core. Consequently, the corresponding photocurrent is enhanced.

These novel fibre structures offer a unique possibility for constructing an optoelectronic functional fabric because the fibres are

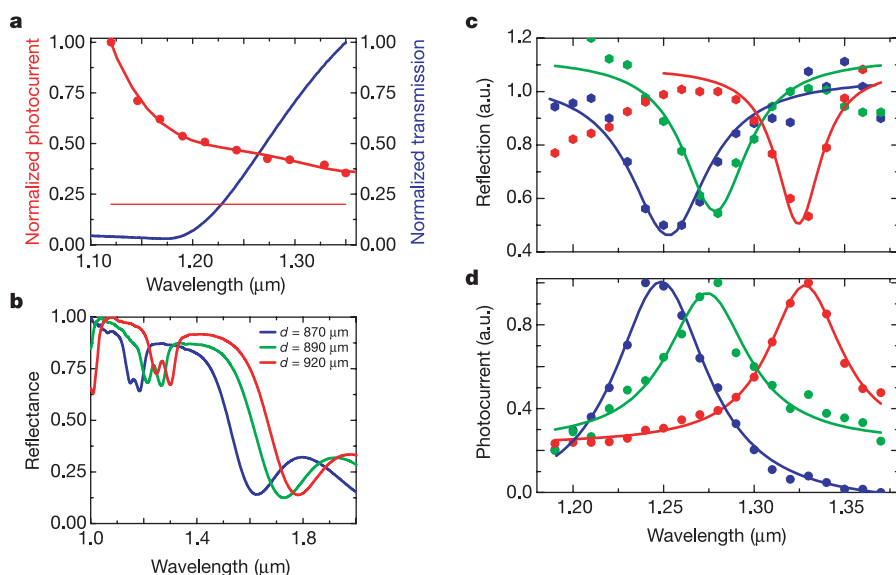


Figure 3 Results of optical and electrical measurements performed on the device fibres. **a**, The red curve is the normalized electrical current measured when illuminating a 890- μm two-electrode fibre, not equipped with a multilayer structure. The horizontal red line indicates the dark current level. The illumination was derived from a tunable OPO (coherent) and the wavelength was swept while keeping the optical power constant. The blue curve shows the normalized transmission of a bulk AST-Sn glass sample.

b, FTIR-measured spectra of device fibres with 870, 890 and 920- μm outer diameter. **c**, Measured back-reflected light power from the same three device fibres when illuminated with a tunable OPO laser beam. **d**, Simultaneously measured photocurrent through the device fibre. Note that at the location of the resonance, the back-reflection is diminished (light is admitted into the fibre and reaches the photoconductive core) while the corresponding photocurrent, consequently, is enhanced.

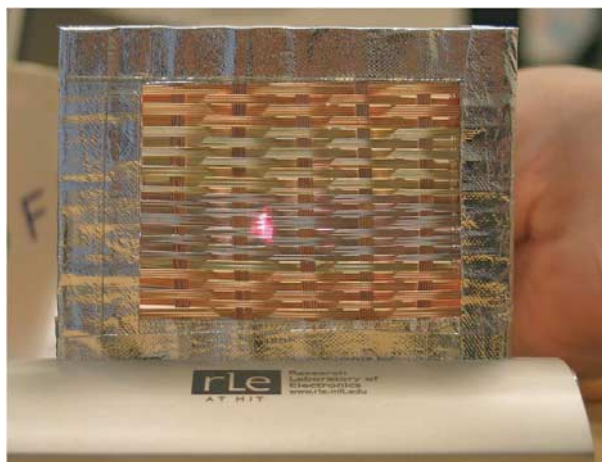


Figure 4 A woven spectrometric fabric. The fabric consists of interleaved threads, 400 to 500- μm -thick, chosen from our device fibres. The visual appearance of the fabric, specifically the range of exhibited colours, is a result of the tight control exerted over the optical properties of the produced fibres.

both flexible and mechanically tough, and can thus be woven, as shown in Fig. 4. The fibres chosen to be used in this fabric are of slightly different diameters (400–500 μm), and hence exhibit different bandgap positions, and resonant cavity wavelengths, manifested in their colours (corresponding to primary photonic bandgaps). Furthermore, interesting device applications follow not only from the ability to engineer the single-fibre properties, but also from the specifics of fibre arrangements into larger assemblies. For example, in constructing a two-dimensional optical detector array, a desired resolution of $N \times N$ pixels per unit area would require N^2 point (dimensionality zero) detection elements. A woven fabric, on the other hand, provides a grid structure, out of linear (dimensionality one) fibres, which in turn can be used to localize an illumination point on a surface—but with detection elements of only order N . Moreover, by overlapping layers of this fabric, one could even ascertain the direction of incoming illumination. These fibres hence enable the realization of large-area optoelectronic functional surfaces. Thus, with the ability to interface materials with widely disparate electrical and optical properties in a fibre, achieve sub-micrometre features and realize arbitrary geometries over extended fibre lengths, we present the opportunity to deliver novel semiconductor device functionalities at fibre-optic length scales and cost. □

Methods

Glass synthesis

The amorphous semiconductor glass, $\text{As}_{40}\text{Se}_{50}\text{Te}_{10}\text{Sn}_5$, is prepared from high-purity As, Se, Te and Sn elements using sealed-ampoule melt-quenching techniques. The materials were weighed and placed into a quartz tube under a nitrogen atmosphere. The tube was heated to 330 °C for an hour at a rate of 1 °C min^{-1} under vacuum to remove surface oxides, and cooled to room temperature (23 °C) at the same rate. The ampoule was formed by sealing the tube under vacuum ($\sim 10^{-5}$ torr). It was then heated to 800 °C at a rate of 2 °C min^{-1} in a rocking furnace, while being held vertical, for 24 h and then rocked for 6 h to increase mixing and homogenization. The glass liquid was cooled to 600 °C in the furnace, and then quenched in water. Subsequently, it was annealed for 30 min near the glass transition temperature, $T_g = 180$ °C, before being cooled gradually to room temperature. Using this procedure, mechanically stable glass rods with diameter 12 mm and length 18 cm were obtained.

Preform preparation and fibre drawing

The metal-core polymer-cladded fibre strands were obtained by drawing a preform with a 5-mm Sn (99.75% purity) core and 7.5-mm PES cladding. The preform was consolidated in a vacuum oven at 260 °C, and then drawn at ~ 305 °C in a vertical tube furnace. Both ends of the preform were sealed with polymer to confine the metal during the consolidation and drawing processes. Metres of uniform metallic fibres with outer diameters from 500 μm to 1.2 mm were successfully drawn and sectioned into strands. Sixty of these Sn strands were used in the construction of a macroscopic preform that yields the current hybrid fibre after drawing.

The dual electron-photon transport fibre preform was then assembled by wrapping a PEI film, coated on both sides with a layer of As_2Se_3 , around a pyrex tube having 16-mm outer diameter. Specifically, a 2.6- μm -thick layer of As_2Se_3 was evaporated uniformly onto both sides of a 20-cm-wide, 8- μm -thick, 1-m-long PEI film. An array of Sn strands were positioned around the outer surface of the multilayer structure by using a polymer solution of 20% PES, 80% N,N-dimethylacetamide. The resulting preform was then consolidated in a vertical rotating furnace at a temperature of 260 °C and a pressure of 10^{-3} torr. In the furnace, the preform longitudinal axis is held vertically and a zone-refining heating process is carried out along the preform length. After consolidation, the preform was immersed in a liquid HF bath for 3 h to selectively etch away the pyrex tube in the centre, leaving a hollow core. The finalized preform was then drawn under the following conditions: a temperature of 302 °C, a downfeed speed of 0.003 mm s^{-1} , and a capstan speed of 1 m min^{-1} .

The spectroscopic fibre preform consists of an $\text{As}_{40}\text{Se}_{50}\text{Te}_{10}\text{Sn}_5$ rod, having diameter 12 mm and length 18 cm, contacted by four Sn conduits, and surrounded by a quarter-wave $\text{As}_2\text{Se}_3/\text{PEI}$ multilayer mirror structure with a $\lambda/2$ PEI cavity and a protective PES cladding. The preform, 20 mm in diameter and 30 cm in length, was consolidated in a three-zone horizontal tube furnace while rotating the preform along its axis. Subsequently, the preform was drawn in a three-zone vertical tube furnace at a top zone temperature between 185 and 230 °C and a middle zone temperature of 295 °C with a tension of 500 g. A capstan speed of 0.7–3 m min^{-1} produces a fibre of a diameter between about 1,200 and 500 μm and a length of several hundred metres.

Optical characterization

For optical characterization, we used a Verdi10 (Coherent) laser to pump a Ti-S femtosecond laser (Mira 900, Coherent). The femtosecond laser beam was then down-converted using an OPO (Mira OPO, Coherent). The OPO beam size was adjusted using a pinhole before passing through a beam splitter to allow collection of back-reflected light from the fibre. The beam emerging from the beam splitter was focused onto the outer surface of the fibre using a $\times 5$ microscope objective ($\text{NA} = 0.1$). The optical power incident on the fibre surface was maintained at 30 mW, using a variable optical attenuator, while the wavelength of the laser beam was swept. Back-reflected light through the beam splitter was detected using a Newport InGaAs photodetector (model 818-IG). For simultaneous electrical characterization, we measured the current flowing through the fibre electrodes using a pico-ampere meter/d.c. voltage source (Yokogawa/Hewlett Packard 4140B). The d.c. voltage difference applied to the two fibre electrodes was 50 V. At each wavelength, the incident optical power was adjusted, the electrical current was recorded, and the back-reflected light power measured.

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Control of ion selectivity in potassium channels by electrostatic and dynamic properties of carbonyl ligands

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Potassium channels are essential for maintaining a normal ionic balance across cell membranes. Central to this function is the ability of such channels to support transmembrane ion conduction at nearly diffusion-limited rates while discriminating for K⁺ over Na⁺ by more than a thousand-fold. This selectivity arises because the transfer of the K⁺ ion into the channel pore is energetically favoured, a feature commonly attributed to a structurally precise fit between the K⁺ ion and carbonyl groups lining the rigid and narrow pore¹. But proteins are relatively flexible structures^{2,3} that undergo rapid thermal atomic fluctuations larger than the small difference in ionic radius between K⁺ and Na⁺. Here we present molecular dynamics simulations for the potassium channel KcsA, which show that the carbonyl groups coordinating the ion in the narrow pore are indeed very dynamic ('liquid-like') and that their intrinsic electrostatic properties control ion selectivity. This finding highlights the importance of the classical concept of field strength⁴. Selectivity for K⁺ is seen to emerge as a robust feature of a flexible fluctuating pore lined by carbonyl groups.

Selective conduction of K⁺ is conferred by the narrowest region of the pore formed by the backbone carbonyl groups of the residue sequence TTVGYG, which is highly conserved among all known potassium channels (Fig. 1)^{5–7}. Although equilibrium and non-equilibrium aspects must both be taken into consideration to address this question completely, the observed selectivity of KcsA for K⁺ can largely be explained thermodynamically: partitioning of the more strongly solvated Na⁺ ions into the narrow pore is unfavourable^{8–10}, reflected in a difference between the free energy of K⁺ and Na⁺ in the pore and in the bulk solution

$$\Delta\Delta G(K^+ \rightarrow Na^+) = [(G_{\text{pore}}(Na^+) - G_{\text{bulk}}(Na^+)) - (G_{\text{pore}}(K^+) - G_{\text{bulk}}(K^+))] \quad (1)$$

that is larger than zero. Ion-flux measurements^{11–13} indicate that the relative free energy of selectivity $\Delta\Delta G$ is of the order of 5–6 kcal mol^{−1} for K⁺ channels. This preference for K⁺ ions is usually explained by pointing out that the channel can compensate for the desolvation of a cation of the correct radius like K⁺ because it fits snugly into the narrow pore, whereas a sufficiently favourable interaction is not possible in the case of a smaller ion such as Na⁺ (refs 1, 5, 7). This explanation is consistent with the observation that the selectivity filter in the X-ray structure of the KcsA channel is well adapted to coordinate K⁺ (refs 5, 7). However, the atomic radii of K⁺ and Na⁺ differ only by 0.38 Å (ref. 14), so the snug-fit mechanism requires the selectivity filter to rigidly retain a precise (sub-ångstrom) geometry to discriminate between these two cations, even though proteins are 'soft materials' displaying significant structural flexibility^{2,3,15}. The crystallographic thermal B-factors of the KcsA X-ray structure determined at 2.0 Å resolution are

indicative of root-mean-square (r.m.s.) fluctuations of the order of about 0.75 Å (see also Supplementary Information)⁷, in general accord with the structural fluctuations seen in molecular dynamics (MD) simulations of the KcsA channel^{16–22}. The experimental observation that K⁺ is needed for the overall stability of the channel structure^{23,24} further supports the notion of a structurally flexible pore. In fact, the diameter of some regions of the pore in the X-ray structure is smaller than the size of K⁺ (ref. 7), so flexibility and fluctuations seem to be essential for rapid conduction¹⁹. MD free-energy perturbation (FEP) computations help to elucidate the origin of energetic factors in dynamic structures^{16–19}, which in the case of a KcsA channel with full structural flexibility yielded ion selectivity in agreement with experimental estimates^{11–13}, despite atomic fluctuations of the order of ~1.0 Å r.m.s. (Fig. 1). Taken together, these observations indicate that a snug structural fit of K⁺ inside the narrow and rigid pore is not the origin of the ion selectivity seen in potassium channels.

An alternative explanation is that selectivity might arise locally, from the intrinsic physical properties of the ligands coordinating the ions passing through the channel²⁵. The most important local interactions are the very strong electrostatic attraction and core repulsion between the cation and the nearest carbonyl groups, and the moderate electrostatic repulsion between the coordinating carbonyl groups themselves. To probe their role in achieving ion selectivity, we monitor changes in $\Delta\Delta G$ upon artificially disrupting these interactions. The effect of turning off the electrostatic interaction between the carbonyls on $\Delta\Delta G$ is particularly informative because it is associated with the dynamical properties of the coordination shell (that is, $\Delta\Delta G$ should not be affected if the coordination structure is rigid). In the following, we compare and contrast the results from FEP computations with and without carbonyl electrostatic repulsion in the KcsA channel with those

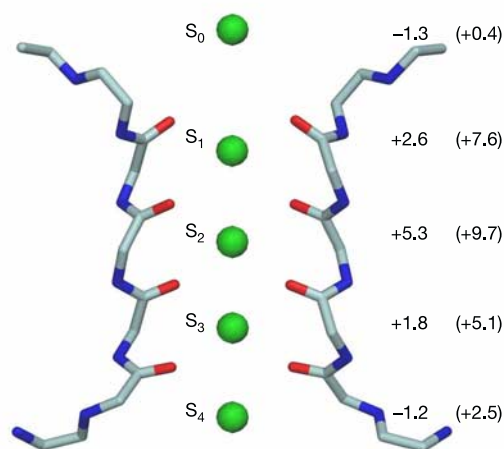


Figure 1 Schematic structure of the cation binding sites in the selectivity filter of the KcsA channel⁷. Only two subunits are depicted for clarity. The extracellular side is on the top and the intracellular side is at the bottom. Results from both X-ray crystallography⁷ and MD free-energy simulations¹⁹ show that five specific cation binding sites (S₀ to S₄) are disposed along the narrow pore of the KcsA K⁺ channel. The cation positions are represented (green) and the carbonyl oxygen group of residues Thr 75, Val 76, Gly 77 and Tyr 78 are shown explicitly (red). Computational studies show that ion movement through the channel takes place in a concerted way, as the K⁺ in the pore undergo hopping transitions between stable multi-ion configurations: [S₃, S₁] ↔ [S₄, S₂, S₀] ↔ [S₄, S₂] ↔ [S₃, S₁] (ref. 20), consistent with structural data^{6,7}. Only configurations in which the cations are separated by one water molecule are allowed. The numbers adjacent to the binding sites are the $\Delta\Delta G$ (in kcal mol^{−1}) obtained from FEP computations based on the KcsA channel in a fully solvated lipid membrane (see Methods). Similar computations based on a frozen channel structure perfectly adapted to K⁺ indicate that it is very selective (numbers in parentheses).

obtained in liquid N-methylacetamide (NMA), a generic model of the protein backbone, and valinomycin, a cationic membrane carrier exhibiting a very high selectivity for K^+ over Na^+ (refs 26, 27). The FEP calculations for the KcsA channel are focused on the site S_2 , located near the middle of the narrow pore, which is the most selective (Fig. 1). The results are reported in Table 1.

Removing the carbonyl–carbonyl interaction in a fully flexible KcsA channel annihilates the selectivity of the site S_2 , which then becomes favourable for Na^+ . The relative free energy, originally unfavourable for Na^+ by $5.3 \text{ kcal mol}^{-1}$, becomes favourable by $-2.9 \text{ kcal mol}^{-1}$ with a net loss of $8.2 \text{ kcal mol}^{-1}$ in selectivity when the repulsive interaction between the backbone carbonyl is turned off (Table 1). *Ab initio* computations on model systems indicate that non-additive electronic polarization (neglected in the present pair-wise all-atom force field)²⁸ would make the free-energy contribution arising from carbonyl–carbonyl repulsion even more unfavourable. Very strong positional energy restraints ($30 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) must be applied to the KcsA atoms to maintain a selectivity of about 5 to 6 kcal mol^{-1} in the absence of the carbonyl–carbonyl repulsion, essentially freezing the channel. The magnitude of the free-energy change in liquid NMA ($10.5 \text{ kcal mol}^{-1}$) is very similar to that found in the fully flexible KcsA channel, suggesting that the coordination of the ions in the selectivity filter of KcsA is indeed very dynamical and ‘liquid-like’. Consistent with this view, the width of the main peak in the radial distribution between the cation and the surrounding oxygen ligands shown in Fig. 2 is similar for KcsA and liquid NMA (of the order of $1.0 \text{ \AA}$).

To further assess the importance of protein rigidity relative to the local interactions, additional FEP calculations were performed, keeping all channel atoms fixed except those forming the selectivity filter (that is, the backbone atom of residues Thr 74 to Asp 78). In particular, the aromatic side chains suggested to play an essential role in the selectivity (that is, Tyr 78, Trp 67, Trp 68)⁵ were kept frozen at their X-ray structure positions. Removing carbonyl repulsion in the selectivity filter results in a loss of almost 6 kcal mol^{-1} of selectivity for K^+ over Na^+ (Table 1), despite the frozen protein surrounding the selectivity filter. Although most conserved residues are essential for the overall stability of the three-dimensional fold (within $\sim 1 \text{ \AA}$), the FEP calculations show that the architectural sub-angstrom rigidity of the protein conferred by the residues surrounding the selectivity filter is not a key factor in making the channel selective for K^+ over Na^+ .

Despite its large impact on the relative free energy of solvation of cations in the pore, the carbonyl–carbonyl ligand repulsion has a moderate influence on pore structure. On average, the repulsion between the eight carbonyl groups renders the system 14 kcal mol^{-1} less stable when Na^+ occupies the S_2 site than when K^+ is present.

Table 1 Importance of carbonyl–carbonyl repulsion on free energy

System	$\Delta\Delta G$ with all interactions (kcal mol^{-1})	$\Delta\Delta G$ with no repulsion (kcal mol^{-1})	Loss in ion selectivity (kcal mol^{-1})
Fully flexible KcsA	5.3	-2.9	8.2
Fully frozen KcsA	9.7	9.7	0.0
Restrained KcsA	8.6	5.9	2.7
Partly frozen KcsA	6.7	0.9	5.8
Liquid NMA	1.6	-8.9	10.5
Valinomycin	8.8	3.9	4.9

All calculations with KcsA concern exclusively the S_2 binding site and are based on the X-ray structure PDB id 1K4C. Fully flexible KcsA, all-atom MD/FEP with fully flexible KcsA embedded in DPPC membrane. Fully frozen KcsA, all-atom MD/FEP with KcsA embedded in DPPC membrane with all channel atoms frozen in the X-ray position. Restrained KcsA, all-atom MD/FEP with KcsA embedded in DPPC membrane with all non-hydrogen channel atoms submitted to a harmonic restraint of $30 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ relative to the X-ray position, yielding RMS fluctuations of $0.11 \text{ \AA}$ for the backbone of the selectivity filter. Partly frozen KcsA, all-atom MD/FEP with KcsA embedded in DPPC membrane allowing motions only for the backbone atoms of the selectivity filter (residues Thr 74 to Asp 78), all other channel atoms are frozen in the X-ray position. Liquid NMA, relative solvation free energy between liquid water and liquid NMA. Valinomycin, relative free energy of ions bound to valinomycin solvated in ethanol.

This difference is much smaller than the large interaction energy between the cation and its surroundings (roughly -150 and $-170 \text{ kcal mol}^{-1}$ for K^+ and Na^+ , respectively). The local coordination structure is thus controlled by the strong ion–carbonyl interactions, with K^+ as well as Na^+ being well-coordinated in the flexible and fluctuating pore; see the radial distribution function between the central cation (K^+ or Na^+) and its coordinating oxygens (Fig. 2). In fact, the average structure is not strongly affected when removing the electrostatic repulsion between the carbonyl groups; for example, the coordination numbers with and without repulsion are quite similar (Fig. 2). The carbonyl–carbonyl repulsion thus has little effect on the pore radius, but instead influences ion-binding energetics. This is in accord with behaviour generally seen in flexible systems undergoing thermal fluctuations (for example, a liquid), where the harshest and strongest interactions dictate the average structure while weaker interactions modulate thermodynamics (for example, the core repulsion and London dispersion in a van der Waals liquid)²⁹.

The ion selectivity of valinomycin is higher than that of KcsA and liquid NMA, although the trends are qualitatively similar (Table 1). As indicated by the narrow width of the radial distribution function (Fig. 2), this cyclic ionophore is more rigid and less able to adapt to coordinate Na^+ than KcsA or liquid NMA as a result of its high covalent connectivity (only three chemical bonds separate each carbonyl group from its neighbours). K^+ is coordinated by six carbonyl oxygen atoms, whereas Na^+ is coordinated by only four carbonyl oxygens (Fig. 2), which increases ion selectivity. In contrast, the selectivity filter of KcsA and liquid NMA are more flexible

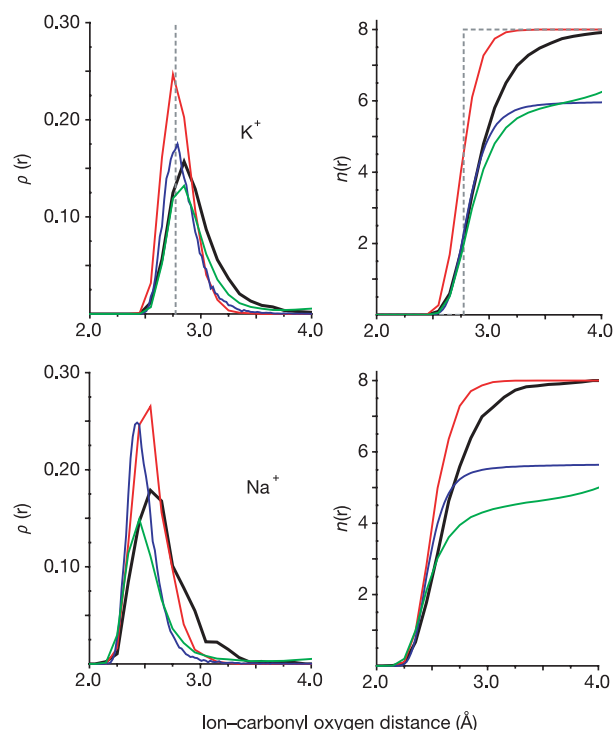


Figure 2 Ion–carbonyl oxygen pair correlation function $\rho(r)$ and coordination number $n(r)$ in different environments. The results for the S_2 binding site of the KcsA selectivity filter with all interactions (solid black line) and without the carbonyl–carbonyl repulsion (solid red line) are shown for K^+ (top) and Na^+ (bottom). For comparison, the corresponding quantities are shown for the X-ray structure (dotted black line), liquid NMA (solid blue line) and in valinomycin (solid green line). The shift in the position of the maximum in the radial distribution for both ions in the selectivity filter of KcsA when carbonyl repulsion is excluded is less than $0.1 \text{ \AA}$, much smaller than the width of the main peak ($\sim 1.0 \text{ \AA}$). In comparison, the shift in the peak position is about $0.32 \text{ \AA}$ between K^+ and Na^+ when all interactions are included.

than valinomycin. In liquid NMA, K^+ and Na^+ are coordinated by six carbonyl oxygens whereas they are coordinated by six to eight oxygens in the binding site S_2 of KcsA. The coordination structure is liquid-like in both cases, but selectivity is more pronounced for the site S_2 of KcsA than for liquid NMA (see Supplementary Information). Further analysis with a simple model suggests that one mechanism for increasing selectivity in a flexible structure is to increase the number of coordinating ligands surrounding the cation (see below).

Although this is not the underlying mechanism of selectivity, the pore is nonetheless structurally well-adapted to coordinate K^+ with minimal structural strain. For example, the peak in the K^+ –oxygen radial distribution function corresponds to the X-ray structure (Fig. 2). The adaptation of the protein surrounding the selectivity filter is perhaps best illustrated by considering the results of *ab initio* geometry optimization of K^+ in the central binding site S_2 . Representing the four subunits forming the binding site as glycine dipeptides, the optimized *ab initio* geometry is seen to depart by 0.28 Å r.m.s. from the high-resolution X-ray structure of KcsA. Such a relatively small deviation (in the absence of the rest of the protein) suggests that the channel has evolved to optimize the location and structure of the ligands coordinating K^+ . But selectivity would be highly sensitive to the sub-ångstrom precision of the selectivity filter if such structural adaptation translated into structural rigidity: additional *ab initio* calculations show that deviation of the four subunits from the optimized configuration by a mere 0.15 Å would be sufficient to abolish the preference for K^+ if the structure were static. Remarkably, thermal fluctuations have the

ability to protect the selectivity of the pore for K^+ against such minor structural changes. To illustrate this unexpected feature of a dynamical fluctuating pore, a reduced model limited to the cation-binding site S_2 was considered. This simple model, comprising only 37 atoms, is depicted in Fig. 3a (see legend for details). Similar FEP calculations were repeated, imposing restraints on the atoms of the model to control the magnitude of atom fluctuations in the system. The dependence of $\Delta\Delta G$ on the magnitude of the allowed r.m.s. atomic fluctuations is plotted in Fig. 3b. As expected, the free energies of selectivity are quite similar with and without carbonyl repulsions if atomic fluctuations smaller than 0.1 Å are permitted. But when the repulsion between the carbonyls is removed, the site becomes progressively more selective for Na^+ as the flexibility of the model increases. To ascertain the importance of the geometry of the binding site, FEP calculations were repeated using a model of S_2 optimized for coordinating Na^+ as a reference structure. As shown in Fig. 3b, this binding site is very favourable for Na^+ as long as the system is kept rigid and only atomic fluctuations smaller than 0.3 Å are allowed. But this enforced binding preference is destroyed as thermal fluctuations become larger, with selectivity for K^+ over Na^+ restored as thermal fluctuations of ~ 0.7 Å are allowed, effectively ‘rescuing’ the intrinsic binding propensity of the site. Selectivity is thus clearly a robust feature by virtue of the flexible and fluctuating

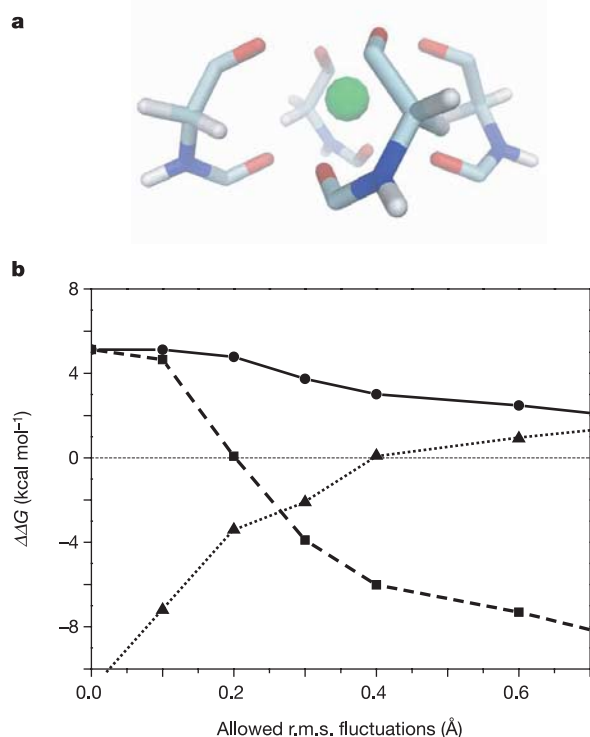


Figure 3 Selectivity of a model of the KcsA binding site S_2 as a function of flexibility. **a**, The structure of the model used to mimic the site S_2 is shown. **b**, The results of the FEP calculations using the X-ray structure of KcsA⁷ as a reference with all interactions (solid line with circles) and excluding the carbonyl–carbonyl repulsion (dashed line with squares) are shown. The results of the FEP calculations using a reference structure optimized to best-coordinate a smaller cation such as Na^+ ion in the binding site (obtained via energy minimization in vacuum) are also shown (dotted line with triangles). In all cases, the maximum r.m.s. fluctuations were controlled using a flat-bottomed steep harmonic potential imposed on all the non-hydrogen protein atoms of the model.

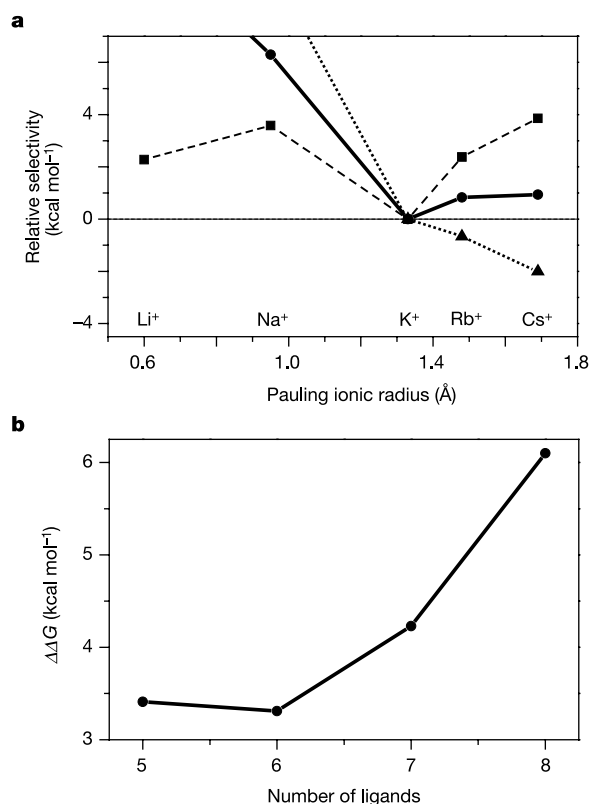


Figure 4 Intrinsic selectivity of a simple model of freely fluctuating carbonyl-like dipoles. **a**, The variations of $\Delta\Delta G$ as a function of ionic radius are shown. The calculations are based on the FEP computations done on a simple model of one cation surrounded by eight carbonyl-like dipoles (comprising two atoms) with the oxygen atoms allowed to move freely within a sphere of radius 3.5 Å. Different cases are illustrated: cation surrounded by eight dipoles of 3.0 debye (solid black line with circles); eight dipoles of 4.2 debye (dashed line with squares) and eight dipoles of 1.8 debye (dotted line with triangles). The results of the FEP calculations for the five cations are plotted using standard ionic radii¹⁴ (the $\Delta\Delta G$ values for the different cases were shifted to bring K^+ to zero). **b**, The dependence of $\Delta\Delta G$ on the number of surrounding carbonyl-like dipoles is illustrated. The selectivity is strongly influenced by the number of carbonyl–carbonyl repulsive pairs (that is, $n(n-1)/2$ for n carbonyl groups).

nature of the system, with carbonyl–carbonyl repulsion ensuring the system's selectivity for K^+ binding.

The present analysis shows that ionic selectivity in K^+ channels is primarily determined by the intrinsic physical properties of the ligands coordinating the cation in the binding site, rather than by the precise sub-ångström geometry of the carbonyl oxygens lining a rigid pore. While this perspective contrasts sharply with structure-based views of ionic selectivity in K^+ channels^{1,5,7}, it shares some of the underlying principles of treatments that identify the strength of the electric field arising from the ligands coordinating the cation in a binding site as a key factor in determining selectivity⁴. In this context, we assess the magnitude of the ligand dipoles required to maintain a robust ionic selectivity in a flexible system undergoing thermal fluctuations. We consider a simple model with eight carbonyl-like ($C=O$) dipoles surrounding a central cation fluctuating freely inside a sphere of 3.5 Å radius. The FEP calculations depicted in Fig. 4 (see legend for details) reveal that such a simple system is naturally selective for K^+ if the ligand dipoles have a magnitude of about 3 debye, a value that is very close to the dipole moment of the protein backbone carbonyl group²⁸. This result further demonstrates that significant selectivity for K^+ over Na^+ is possible without any need for architectural rigidity that would prevent the coordinating ligands from collapsing onto a smaller cation.

The main findings from the simple model are consistent with the existence of the conserved TTVGYG motif, which is structurally needed to fold the polypeptide chain into the ion-conducting conformation with the main-chain carbonyl oxygens oriented towards a central pore axis⁵. The selectivity displayed by the simple model is sensitive to the number of coordinating groups; significant selectivity for K^+ over Na^+ is obtained only when eight freely fluctuating carbonyl-like dipoles are present (Fig. 4b). This explains the origin of the low K^+ selectivity in liquid NMA with six nearest neighbours in the first solvation shell (Fig. 2). It also suggests that the three-dimensional fold of the selectivity filter (Fig. 1) might serve to enhance the local 'concentration' of fluctuating carbonyl groups within a small region. The simple model with freely fluctuating carbonyl dipoles leaves out any factors related to protein geometry and rigidity, yet displays qualitative trends in good accord with experimental observations: the selectivity for K^+ over Na^+ is of the order of 5–6 kcal mol⁻¹, whereas the selectivity over Rb^+ and Cs^+ is somewhat smaller^{9,10}. The flexible binding site remains optimal for K^+ as long as the coordinating ligands have a dipole roughly between 2.5 and 4.5 debye, that is, are carbonyl-like. Increasing the magnitude of the dipoles beyond 4.5 debye favours smaller cations, whereas decreasing the dipoles has the opposite effect (Fig. 4a). However, decreasing the magnitude of only four out of eight carbonyl dipoles does not alter the selectivity significantly, in accord with experiments introducing a backbone ester carbonyl mutation in the selectivity filter³⁰. These observations are consistent with the classical concept of 'field strength' developed by Eisenman⁴, although the present analysis incorporates also the influence of thermal atomic fluctuations and ligand–ligand repulsion.

A sharp departure from eight identical carbonyl-like fluctuating ligands is required to make the site favourable for Na^+ over K^+ ; for example, it becomes selective for Na^+ when the magnitude of the dipoles is ~7 debye. One possible way of achieving Na^+ selectivity (although there are others) would be the introduction of charged residues forming a salt bridge directly into the pore. It is intriguing that the amino acids identified to be essential for the selectivity of Na-channels include the highly conserved DEKA locus from four different protein domains^{10,31}. Selectivity for K^+ or for Na^+ clearly requires different chemical functionalities to coordinate the cation favourably, in accord with the observation that no biological channels selective to Na^+ appear to have evolved by refining the geometry of a KcsA-like pore lined by backbone carbonyl groups¹⁰. □

Methods

Models

The atomic system and simulation methodology have been described elsewhere¹⁹. Briefly, the total number of atoms in protein simulations is slightly above 40,000 (KcsA, 112 dipalmitoyl phosphatidylcholine, 6,778 water molecules, three K^+ ions in the pore, six K^+ and 21 Cl^- ions in the bulk solutions). The high-resolution structure of the channel was used⁷. The X-ray coordinates were relaxed by less than 0.12 Å to remove any strain in the reference structure. The simple model system for the S_2 binding site occupied by a K^+ (37 atoms with one cation) was taken directly from this structure (Fig. 3a). The corresponding model system for the S_2 binding site occupied by a Na^+ was refined by energy minimization in vacuum to generate an optimal coordination for this cation; the resulting structure retained the overall four-fold symmetry but was slightly collapsed onto the smaller cation, deviating from the original structure with K^+ by about 0.51 Å. Two different starting configurations of KcsA embedded in a solvated lipid membrane (' S_0 : S_2 : S_4 ' and ' S_1 : S_3 :cavity') were prepared using the previously published protocols¹⁹. The FEP computations on the binding sites of KcsA were carried out using those configurations, alchemically transforming one cation at a time. FEP simulations of ion solvation in liquid NMA were carried out using a cubic box of 150 NMA molecules and one ion. FEP simulations of ion selectivity in valinomycin were carried out using a cubic box of 225 ethanol molecules. See Supplementary Information for more detail about the NMA and valinomycin systems.

Computational procedure

All MD simulations and FEP computations were carried out using a modified version of the program CHARMM³². The total length of simulation preceding the FEP computations was over 1.8 ns. The resulting structure was used to perform the FEP calculations in each binding sites (Fig. 1). The variation in $\Delta\Delta G$ along the pore is associated with the hydration of the cation in the different binding sites (Fig. 1); similar results¹⁹ were obtained using the X-ray structure at lower resolution⁵. The binding sites S_1 and S_3 near the ends of the selectivity filter are less selective (cations in those sites are not completely dehydrated, but maintain some contacts with one or two water molecules) whereas a cation in S_2 is completely dehydrated and coordinated by eight backbone carbonyl oxygens (from Gly 77 and Val 76). To remove the carbonyl–carbonyl repulsion, the Coulomb interactions between those atoms were simply skipped from the main loop in the total energy and forces calculation (this procedure differs from a direct scaling of the partial charges of the carbonyl to zero, because all the electrostatic interactions of the carbonyl with the ions, water molecules and with the remainder of the system remain unaffected). The selectivity filter simulated with and without carbonyl repulsion deviate only by 0.5 Å from one another (the average r.m.s. relative to the X-ray structure is 0.7 Å with repulsion and 0.6 Å without repulsion).

For each of the FEP computation (KcsA, valinomycin, liquid NMA, and the simple models used in Figs 3 and 4), the forward and backward directions free-energy perturbation ($K^+ \leftrightarrow Na^+$) had values of coupling parameter λ varying from 0 to 1 by increments of 0.05 for a total 1.1 ns. All calculations with the frozen or restrained channel structure were carried out according to the same FEP protocol (except that all or some channel atoms are kept fixed at all times) as described previously¹⁹. The potential function of the ions was parameterized to yield an accurate description of solvation in bulk water and liquid amides. The Lennard–Jones parameters for cations were adjusted to reproduce the experimental free energies of solvation as described previously¹⁹. Solvation free energies in water were calculated using FEP. For the rest of the atoms, the standard PARAM-22 force field was used²⁸. To assess the importance of non-additive forces associated with induced electronic polarization on the FEP computations, *ab initio* computations were performed for a model system of two NMA molecules coordinating one cation (K^+ or Na^+ ; the results are given in the Supplementary Information).

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Low marine sulphate and protracted oxygenation of the Proterozoic biosphere

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Progressive oxygenation of the Earth's early biosphere is thought to have resulted in increased sulphide oxidation during continental weathering, leading to a corresponding increase in marine sulphate concentration¹. Accurate reconstruction of marine sulphate reservoir size is therefore important for interpreting the oxygenation history of early Earth environments. Few data, however, specifically constrain how sulphate concentrations

may have changed during the Proterozoic era (2.5–0.54 Gyr ago). Prior to 2.2 Gyr ago, when oxygen began to accumulate in the Earth's atmosphere^{2,3}, sulphate concentrations are inferred to have been <1 mM and possibly <200 μ M, on the basis of limited isotopic variability preserved in sedimentary sulphides⁴ and experimental data showing suppressed isotopic fractionation at extremely low sulphate concentrations^{1,5}. By 0.8 Gyr ago, oxygen and thus sulphate levels may have risen significantly^{6,7}. Here we report large stratigraphic variations in the sulphur isotope composition of marine carbonate-associated sulphate, and use a rate-dependent model for sulphur isotope change that allows us to track changes in marine sulphate concentrations throughout the Proterozoic. Our calculations indicate sulphate levels between 1.5 and 4.5 mM, or 5–15 per cent of modern values, for more than 1 Gyr after initial oxygenation of the Earth's biosphere. Persistence of low oceanic sulphate demonstrates the protracted nature of Earth's oxygenation. It links biospheric evolution to temporal patterns in the depositional behaviour of marine iron- and sulphur-bearing minerals⁴, biological cycling of redox-sensitive elements⁶ and availability of trace metals essential to eukaryotic development⁸.

To understand Proterozoic biospheric oxygenation better, we have modified an existing model for C isotope variability⁹ to estimate marine sulphate reservoir size using rates of S isotope variation. These rates are recorded stratigraphically as variation in the S isotope composition of evaporitic gypsum and carbonate-associated sulphate (CAS). Because CAS is routinely trapped in marine carbonates¹⁰ and, in organic-poor sediments, is isotopically buffered against appreciable diagenetic overprints¹¹, it can record the $\delta^{34}\text{S}$ signal of marine sea water even when gypsum is lacking. Furthermore, CAS is a more direct proxy for marine sea water than sedimentary pyrite, the isotopic composition of which is complicated by local reservoir effects and varying fractionations during bacterial sulphate reduction (BSR)¹².

In our model, the isotopic composition of marine sulphate changes in response to imbalances in isotopic fluxes into and out of a non-steady state system (see Methods). In the modern ocean, sulphate concentrations of 28.4 mM effectively buffer the isotopic system from changing at rates exceeding 0.5‰ per Myr, despite large fractionations associated with the modern bacterial sulphur cycle (Fig. 1). Long-term variation in the isotopic composition of

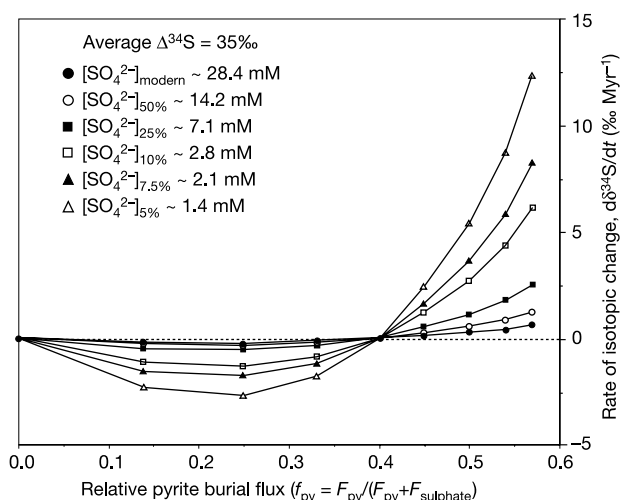


Figure 1 Sensitivity of the marine sulphate system to reservoir size. At average fractionations observed within the modern bacterial sulphur cycle ($\Delta S = 35\text{‰}$), modern reservoir sizes effectively buffer the marine system from the large S isotope shifts that characterize Proterozoic stratigraphic sections, even at moderate to high degrees of pyrite burial ($f_{\text{py}} = 0.2\text{--}0.6$).

marine sulphate from the late Palaeozoic to recent^{11,13–15} can then be explained by $\pm 40\%$ variation in weathering (and/or volcanogenic) fluxes and pyrite burial. Increased pyrite burial drives isotopic compositions to higher (more ^{34}S -enriched) $\delta^{34}\text{S}$ values, and increased weathering results in lower $\delta^{34}\text{S}$. When the sulphate reservoir is small, however, flux imbalances can drive much more rapid isotopic variation, such as that observed in Proterozoic successions^{16–21}.

Samples for CAS analysis were collected from measured stratigraphic sections of the 1.3-Gyr-old Dismal Lakes Group and 1.2-Gyr-old Society Cliffs Formation, Arctic Canada, and extracted via standard methods of acid dissolution and precipitation of CAS as barium sulphate^{11,16}. Both units reveal large, systematic shifts in $\delta^{34}\text{S}$ of up to 12‰ over 300 m in the Society Cliffs and up to 15‰ over 200 m in the Dismal Lakes Group (Fig. 2, and Supplementary Information). Similar patterns of rapid isotopic change are unknown from late Palaeozoic and younger successions but are recorded in both sulphate and sulphide isotopic data from numerous other Proterozoic successions^{16–20}, suggesting that rapid S isotope change is a common, if not universal, feature of Proterozoic oceans. Furthermore, in the Society Cliffs Formation, CAS data show isotopic trends that mimic (with $\sim 3\text{--}5\%$ offset) that of interbedded gypsum, the marine origin of which is constrained by Sr isotope and trace element data²¹. Although the cause is unknown, the isotopic offset is small compared to the magnitude of observed shifts, and the consistency of isotopic trends in gypsum and CAS offers unprecedented corroboration that CAS accurately records changes in marine S isotope composition.

Pb–Pb isotope analysis of a stratigraphic suite of carbonate samples provide an age of $1,204 \pm 22$ Myr for the Society Cliffs and overlying Victor Bay and Athole Point formations (1,750 m total thickness)²¹. If this range, from 1,226 to 1,182 Myr ago, represents the maximum duration of stratal deposition, we can estimate the minimum accumulation rate of the Society Cliffs Formation to be approximately 40 m Myr^{-1} . Similarly, the 1,200-m-thick Dismal Lakes Group is constrained by a combination of radiometric and chemostratigraphic data to have been deposited between 1,300 and 1,270 Myr ago²², again indicating a minimum accumulation rate of 40 m Myr^{-1} . Despite uncertainty in determin-

ing deposition rates for Proterozoic successions, calculated accumulation rates of 40 m Myr^{-1} are similar to minimum accumulation rates ($30\text{--}80 \text{ m Myr}^{-1}$) observed for Palaeozoic carbonate platforms²³. A conservative estimate of $30\text{--}50 \text{ m Myr}^{-1}$ thus provides a reasonable estimate for minimum accumulation rates of shallow marine carbonate successions, even when appropriate time constraints are lacking, and is applied to all model data.

Accumulation rates of $30\text{--}50 \text{ m Myr}^{-1}$ provide minimum rates of S isotope change of $1.4\text{--}2.0\%$ Myr^{-1} and $2.6\text{--}3.8\%$ Myr^{-1} for the Society Cliffs Formation and Dismal Lakes Group, respectively. In terms of our model, these values yield sulphate concentrations of $2.7\text{--}4.5 \text{ mM}$ for the Society Cliffs Formation and $1.5\text{--}2.4 \text{ mM}$ for the Dismal Lakes Group, with higher deposition rates resulting in lower sulphate concentrations. Sulphate concentrations of $1.5\text{--}4.5 \text{ mM}$, or $\sim 5\text{--}15\%$ of modern concentrations, are therefore considered to represent maximum marine sulphate concentrations for the Mesoproterozoic, and are consistent with hypotheses that low-oxygen conditions persisted at least into the Mesoproterozoic^{4,8,24}.

More importantly, our model provides a mechanism for exploring the long-term dynamics of biospheric oxygenation (Fig. 3, Table 1). CAS data from pericratonic, shallow-water carbonates of the 1.7-Gyr-old McNamara Group, Australia, show little systematic pattern of isotopic change, although data reveal several stratigraphic shifts of up to 10‰ over intervals of 50 m (ref. 19, and Supplementary Information). These shifts suggest maximum marine sulphate concentrations between 0.5 and 0.9 mM. Because S isotope compositions would have been extraordinarily sensitive to even minor variation in fluxes at these low concentrations, data scatter probably represents local overprinting of global isotopic signals. Despite limitations of these data, our model results are consistent with sulphate concentrations of $0.5\text{--}2.0 \text{ mM}$ estimated from Fe–S–P flux-based models constructed to explain the persistence of ^{34}S -enriched sulphides at this time²⁵. Combined, CAS and Fe–S–P models suggest that increased biospheric oxygen at 2.2 Gyr ago need not have raised oceanic sulphate concentrations appreciably beyond levels estimated for much of the Archaean.

CAS data from the 1.45-Gyr-old Helena Formation, Belt Supergroup, Montana, show stratigraphic changes in S isotope compo-

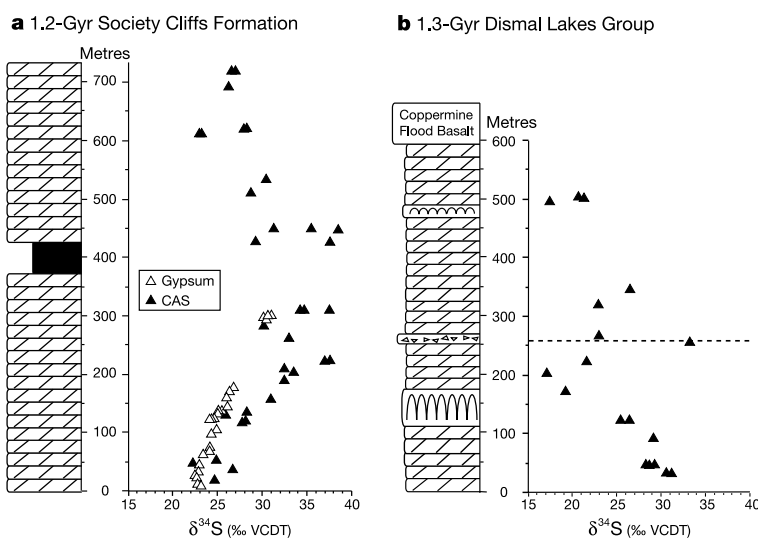


Figure 2 Isotopic composition of CAS from Mesoproterozoic strata. **a**, CAS data from the 1.2-Gyr-old Society Cliffs Formation are similar to S-isotope data from penecontemporaneous gypsum²¹ and show correspondingly large stratigraphic shifts in S-isotope composition. **b**, CAS data from the 1.3-Gyr-old Dismal Lakes Group reveals similarly large isotopic shifts, with strong discontinuity apparent only at a major subaerial

exposure surface (dotted line). All S isotope compositions are expressed in standard delta notation as per mil (‰) deviations from VCDT, with analytical error of $<0.01\%$, calculated from replicate analyses of laboratory standards. See Supplementary Information for tabulated data.

sition of up to 15‰ over 200 m (ref. 19). Similar trends are preserved by sulphides in the 1.47-Gyr-old Prichard and Newland formations, Belt Supergroup^{12,17}, as well as CAS from the 1.3-Gyr-old Dismal Lakes Group, supporting an increase in marine sulphate concentration to approximately 1.5–2.4 mM by this time. Although the paucity of data inhibits detailed reconstruction of marine sulphate concentrations during the Proterozoic, it is clear that sulphate concentrations remained significantly low for at least 900 Myr after initial oxygenation of the Earth's biosphere. By contrast, CAS data from the 1.2-Gyr-old Society Cliffs Formation show stratigraphic changes in S isotope composition that yield estimates of 2.7–4.5 mM sulphate, nearly double that estimated for 1.3-Gyr strata.

In the Neoproterozoic, observed fractionation between coeval sulphate and sulphide increased to ~35‰ (ref. 6), which would have led to increased rates of S isotope change. However, sparse CAS data from the (probably) early Neoproterozoic Vazante Formation, Brazil²⁰, suggest rates of isotopic change as low as 0.75‰ Myr⁻¹—much lower than those observed in the Mesoproterozoic—

suggesting that marine sulphate concentrations may have risen to 6–10 mM, or 20–35% of modern values, by this time. Similarly low rates of isotopic change recorded in interglacial carbonates of the ~740-Myr-old Otavi Group, Namibia (Ombaatjie Formation)¹⁶ suggest sulphate concentrations near 7 mM, or 25% of modern values, may have persisted through much of the Neoproterozoic. In sharp contrast to preglacial and interglacial successions, large and rapid shifts in CAS composition in post-glacial cap carbonates (Rasthof and Maieberg formations) of the Otavi Group¹⁶ suggest marine sulphate concentrations of <1.3 mM and support hypotheses of near complete reduction of the oceanic sulphate reservoir during Neoproterozoic glacial episodes¹⁶. Together, these data suggest that marine sulphate concentrations probably remained low, <35% of modern values, for nearly the entire Proterozoic. A significant rise in biospheric oxygen, and thus oceanic sulphate, may not have occurred until the latest Neoproterozoic (0.54 Gyr), just before the Cambrian explosion, when sulphate levels may have reached 20.5 mM, or 75% of present-day levels²⁶.

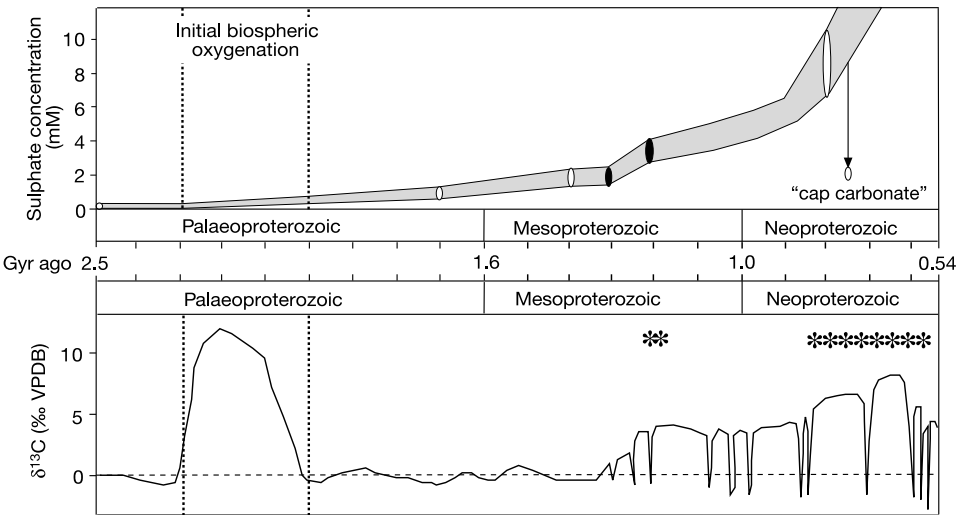


Figure 3 Proterozoic marine sulphate concentrations. Filled ovals mark data from this study; open ovals mark data referred to in the text^{4,16,19,20}. Marine sulphate concentrations probably remained below 2 mM until about 1.3 Gyr ago, rose to ~4.5 mM by 1.2 Gyr ago, and possibly as high as 7–10 mM by the mid-Neoproterozoic, although near-complete reduction of oceanic sulphate may have occurred during Neoproterozoic glacial intervals

(arrow)¹⁶. Increased sulphate concentrations coincide with changes in marine $\delta^{13}\text{C}$ associated with biospheric oxygenation and are marked by the appearance of bedded gypsum (asterisks) in the late Mesoproterozoic and more frequent gypsum deposition in the Neoproterozoic.

Table 1 Marine sulphate concentrations for Mesoproterozoic and Neoproterozoic strata

	Deposition rate, estimated (m Myr ⁻¹)	Observed $\delta\text{S}/\text{dt}$ (‰ Myr ⁻¹)	Inferred $\delta\text{S}_{\text{max}}/\text{dt}$ (‰ Myr ⁻¹)	Max. $[\text{SO}_4^{2-}] \Delta\text{S} = 25$ (mM)	Max. $[\text{SO}_4^{2-}] \Delta\text{S} = 35$ (mM)
~1.7-Gyr McNamara Fm. ¹⁹	30	6.0	60	0.9	*
	50	10.0	100	0.5	
~1.45-Gyr Helena Fm. ¹⁹	30	2.3	26	2.4	*
	50	3.8	38	1.5	
~1.3-Gyr Dismal Lakes Grp	30	2.3	26	2.4	*
	50	3.8	38	1.5	
~1.2-Gyr Society Cliffs Fm.	30	1.4	14	4.5	*
	50	2.0	20	2.7	
~750-Myr Vazante Fm. ²⁰	30	0.8	7.5	*	10.1
	50	1.3	12.5		6.1
~740-Myr Otavi Grp ¹⁶	30	1.1*	10.5	*	7.2
	50	1.8*	17.5		2.8
~740 Myr Otavi Grp ¹⁶	30	6.0†	60	*	1.3
	50	10.0†	100		0.8

Marine sulphate concentrations are calculated from CAS data.
* Reported values are estimates of S isotope change recorded in the Ombaatjie Formation, which was deposited during a Neoproterozoic interglacial period.
† Reported values are estimates of S isotope change in 'cap carbonate' strata, which directly overlie glacial diamicrites and are believed to record Neoproterozoic glacial conditions. See text for details.

Increases in oceanic sulphate in both the mid-Mesoproterozoic and Neoproterozoic are temporally consistent with abrupt changes in the C isotope record (Fig. 3) and inferred increases in biospheric oxygenation^{3,21,27}, and are reflected in the geologic history of marine gypsum deposition. In the presence of available Ca, late Mesoproterozoic sulphate concentrations of 2.7–4.5 mM would require >95% reduction in water volume in order to reach gypsum saturation. If, at this time, Ca availability was limited by excess precipitation of calcium carbonate resulting from elevated carbonate saturation²⁸, an even greater degree of evaporation would be required to reach gypsum saturation. In either case, estimated sulphate concentrations are consistent with rare bedded gypsum in the late Mesoproterozoic and apparent deposition of halite before gypsum²¹. In comparison, lower sulphate concentrations before 1.3 Gyr ago would probably result in only isolated occurrences of gypsum, and more frequent gypsum deposition would not be expected to occur until the Neoproterozoic, when sulphate concentrations may have reached at least 25% of modern values.

Calculations presented here suggest that marine sulphate concentrations, and thus biospheric oxygen levels, though increasing, remained low through at least the Mesoproterozoic, and probably well into the Neoproterozoic. This interpretation is consistent with the possibility of long-term, anoxic deep-ocean conditions during the Proterozoic^{4,8,24}. The data, however, do not specifically indicate the presence of sulphide in the water column. Sulphate concentrations as low as 1–2 mM may have persisted for nearly 1,000 Myr after initial biospheric oxygenation. Increasing sulphate concentrations within an oxygen-deficient deep ocean would have supported the development of widespread sulphidic (euxinic) bottom waters⁴. The ubiquity of sulphidic waters in turn may have affected the ecological and evolutionary development of eukaryotes via limitation of redox-sensitive bioessential nutrients⁸. In this light, further increases in atmospheric and thus oceanic oxygenation, as delineated by our estimates for seawater sulphate, are consistent with independent evidence for eukaryotic diversification in the late Mesoproterozoic and Neoproterozoic. Our model for rates of isotopic change in the marine sulphate reservoir thus provides a window into early Earth oxygenation, and establishes a framework for continued examination of Proterozoic biospheric evolution. □

Methods

Sulphate reservoir size is estimated from the relationship $[d\delta_{\text{sulphate}}/dt] = [F_w(\delta_w - \delta_{\text{py}}) - F_{\text{py}}\Delta S]/M_o$, where F_w is the total input flux of sulphur to the ocean-atmosphere system from weathering, δ represents the isotopic composition of fluxes associated with weathering and sulphate deposition ($\delta^{34}\text{S}$ in ‰ = $[(^{34}\text{S}/^{32}\text{S})_{\text{sample}}/(^{34}\text{S}/^{32}\text{S})_{\text{standard}} - 1] \times 1,000$ relative to the Cañon Diablo Troilite standard), M_o is the mass of sulphur in the oceans as sulphate, and ΔS is the observed fractionation between oxidized and reduced sulphur reservoirs ($\Delta S = \delta_{\text{py}} - \delta_{\text{py}}'$) expressed as a negative number. In the model, F_{py} is allowed to vary, and δ_{py} varies according to the equation $\delta_{\text{py}} = \delta_w - (\Delta S f_{\text{py}})$, where $f_{\text{py}} = F_{\text{py}}/(F_{\text{py}} + F_w)$, representing the relative rate of pyrite burial. Estimates for modern fluxes and their isotopic compositions are as follows: $M_o = 1.3 \times 10^{21}$ g, $F_w = 1.0 \times 10^{14}$ g yr⁻¹, $\delta_w = 6\text{‰}$, $F_{\text{py}} = 6 \times 10^{13}$ g yr⁻¹, $F_{\text{py}}' = 4 \times 10^{13}$ g yr⁻¹, $\Delta S = 35\text{‰}$ (refs 29, 30).

Following from $[d\delta_{\text{sulphate}}/dt] = [F_w(\delta_w - \delta_{\text{py}}) - F_{\text{py}}\Delta S]/M_o$, maximum rates of isotopic change are reached when S input to the oceans approaches zero ($F_w' = 0$) and the standing marine sulphate reservoir is removed as pyrite ($F_{\text{py}}' = F_w$), giving $[d\delta_{\text{max}}/dt] = [F_w\Delta S]/M_o$ or $d\delta_{\text{max}}/dt = \Delta S/T_R$ where ΔS is the average maximum fractionation during BSR and T_R is the residence time for sulphate in the oceans [$T_R = M_o/F_w$]. Sulphate residence time is approximately 13 Myr in the modern ocean²⁹.

In the present study, sulphate reservoir size (in grams S as sulphate) is calculated as $M_o = [F_w\Delta S]/[d\delta_{\text{max}}/dt]$. S isotope trends for late Palaeozoic and younger strata^{11,13,14} indicate that natural variation in the rates and isotopic composition of weathering and pyrite burial fluxes result in rates of isotopic change ($d\delta_{\text{sulphate}}/dt$) that reflect only 3–14% of maximum possible rates of isotopic change ($d\delta_{\text{max}}/dt$). Observed variation in Proterozoic isotopic records is therefore estimated to represent 10% of maximum values. See Supplementary Information for graphical representation of model sensitivities.

In this model, M_o is most sensitive to changes in ΔS , wherein a 10% change in ΔS (~5‰) results in a 0.7 mM change in M_o . In laboratory experiments, single-step, dissimilatory sulphate reduction can achieve fractionations up to 40–45‰, and disproportionation in the oxidative part of the bacterial sulphur cycle can produce fractionations >45‰ (refs 1, 6). These high values, however, are rare in many natural systems⁵. Heavier values typically preserved in the geologic record reflect the integration of

bacterial fractionation during progressive depletion of sulphate in sedimentary pore waters during BSR⁷. In our calculations, we have allowed the average maximum fractionation during BSR (ΔS) to vary with time as indicated by empirical data recorded in Proterozoic strata. Prior to ~850 Myr ago, observed maximum values for ΔS rarely exceeded an average of 25–30‰ (ref. 6); after ~850 Myr ago, when biospheric oxygen levels may have facilitated enhanced fractionations, in part through bacterial disproportionation pathways, observed maximum values for ΔS increase to an average of ~35‰ (refs 6, 29). Although ΔS values <25‰ are not uncommon in the Proterozoic⁶, the presence of high ΔS values (25‰ and 35‰, respectively) suggests that Meso- to Neoproterozoic open marine systems may have supported only limited renewal of sulphate to the site of BSR¹², resulting in heavy values representative of suppressed net bacterial fractionation.

Calculations of M_o are least sensitive to changes in F_w . In our model, a 10% decrease in F_w results in a 0.3 mM reduction in M_o . In the Proterozoic, lower atmospheric oxygen would have resulted in a decrease in the oxidative weathering of sulphate to the oceans. Yet because of uncertainties regarding the magnitude of Proterozoic weathering, we have used modern weathering fluxes to ensure that our calculations reflect maximum values for M_o .

In contrast to ΔS and F_w , the sensitivity of M_o to changes in $d\delta_{\text{max}}/dt$ varies as a natural log with respect to deposition rate. M_o is most sensitive at deposition rates <30 m Myr⁻¹ (~2.3 mM increase for 10 m Myr⁻¹ rate decrease), moderately sensitive at rates of 30–50 m Myr⁻¹ (~0.9 mM per 10 m Myr⁻¹ rate increase), and insensitive at rates >50 m Myr⁻¹ (<0.4 mM per 10 m Myr⁻¹ rate increase). Deposition rates are estimated to be 30–50 m Myr⁻¹, which is consistent with available, though imperfect, radiometric and chemostratigraphic constraints, as well as with average deposition rates calculated for Palaeozoic carbonate platforms²³. Although instantaneous rates of deposition were probably higher, shallow marine carbonate accumulation is typically limited by accommodation space, and stratal thicknesses reflect average subsidence through the duration of sediment accumulation. For epicratonic and pericratonic environments typical of carbonate deposition, much of this time is represented by unconformities that coincide with parasequence and sequence-scale stratigraphic boundaries. Fortunately, lateral tracing of depositional boundaries and the continuity of our preserved isotopic trends suggests that, with rare exceptions, individual unconformities are short in duration compared to the total time represented by the sediment package. Our conservative estimates therefore ensure that our calculations reflect maximum possible values for M_o .

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A new troodontid dinosaur from China with avian-like sleeping posture

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Discovering evidence of behaviour in fossilized vertebrates is rare. Even rarer is evidence of behaviour in non-avian dinosaurs that directly relates to stereotypical behaviour seen in extant birds (avians) and not previously predicted in non-avian dinosaurs^{1,2}. Here we report the discovery of a new troodontid taxon from the Early Cretaceous Yixian Formation of western Liaoning, China. Numerous other three-dimensionally preserved vertebrate fossils have been recovered recently at this locality, including some specimens preserving behavioural information³. The new troodontid preserves several features that have been implicated in avialan origins. Notably, the specimen is preserved in the stereotypical sleeping or resting posture found in extant Aves⁴. Evidence of this behaviour outside of the crown group Aves further demonstrates that many bird features occurred early in dinosaurian evolution^{5,6}.

Theropoda Marsh, 1881
Maniraptora Gauthier, 1986
Troodontidae Gilmore, 1924
Mei long gen. et sp. nov.

Etymology. *Mei* from Chinese, meaning to sleep soundly; *long* from Chinese, meaning dragon.

Holotype. IVPP (Institute of Vertebrate Paleontology and Paleoanthropology, Beijing) V12733, a nearly complete, fully articulated skeleton (Fig. 1).

Locality and horizon. Lujiatun, Shangyuan, Beipiao City, western Liaoning, China; lowest more fluvial, volcanoclastic beds of Yixian Formation, older than 128 and younger than 139 million years⁷.

Diagnosis. *Mei long* is distinguishable from all other troodontids on

the basis of extremely large nares extending posteriorly over one-half of the maxillary tooth row; closely packed middle maxillary teeth; maxillary tooth row extending posteriorly to the level of the preorbital bar; a robust, sub-'U'-shaped furcula; presence of a lateral process on distal tarsal IV; and the most proximal end of the pubic shaft is significantly compressed anteroposteriorly, and extends laterally just ventral to the articulation with the ilium.

Mei long is about 53 cm in length, similar in size to the basal dromaeosaurid *Microraptor zhaoianus* and the basal avialan *Archaeopteryx lithographica*^{8,9}. IVPP V12733 is not an adult, as several cranial sutures are unfused, and although fused, sutures between the neural arches and centra are still apparent on the dorsal vertebrae. However, the holotype is also not a hatchling as caudal and sacral vertebral sutures are not apparent, the parietal is formed from a single element and there is complete fusion of astragalus and calcaneum, indicating that the individual is approaching maturity.

As in *Sinovenator changii* and basal dromaeosaurs⁵, the skull is proportionally small (about 69% of the femoral length), the trunk short and the hindlimbs very long. Long hindlimbs relative to the trunk is a feature correlated with a knee-based avian running mechanism¹⁰; it is also present in the basal troodontids *Sinovenator* and *Sinornithoides*^{11,12}, the basal dromaeosaurid *Microraptor*, and the basal oviraptorosaurian *Caudipteryx*^{5,10}. As in other troodontids^{13,14} the numerous maxillary teeth (approximately 24) are tightly packed anteriorly (Fig. 2a, b). Unlike other troodontids, even the middle teeth lack inter-crown space. Posteriorly, the teeth are more stout and re-curved. As with other troodontids⁵, the internarial bar is strap-like and 'T'-shaped. Additionally, a pneumatopore lies on the posterior quadrate surface; a prominent convexity lies lateral to the foramen magnum on the exoccipital/opisthotic; and the distally expanded pendulous paroccipital processes are vertical and apneumatic. The dentary nutrient foramina lie in a horizontal groove on the labial surface of the dentary as in other troodontids^{13,15}.

As in *Sinovenator changii* the dorsal vertebrae have fan-shaped neural spines and slender transverse processes, but lack pneumatic foramina⁶. The distal caudals are elongate, with a reduced centrum and a sulcus on their dorsal surface as in other troodontids¹². The ilium is short and tapers posteriorly. The pubis is long and proximally thick, but is not mediolaterally compressed as in other troodontids including *Sinovenator changii*. The ischium is fairly short with a distally positioned obturator process and two small processes on the dorsal edge. The tibia has an anteroposteriorly wide proximal end, and a distal end with a thick lateral margin. As in basal dromaeosaurids and *Sinovenator*^{16,17} the feet are subarcotome-tarsalian, and metatarsal III is reduced but still visible on the plantar surface (Fig. 1b). Metatarsal II bears a prominent medial flange and metatarsal IV a lateral one on the ventral surface, producing a longitudinal metatarsal palmar excavation. As in other deinonychosaurs, the second pedal digit is specialized with a hypertrophied claw, but is not developed to the same degree as seen in derived dromaeosaurids^{5,18}.

Mei long differs with respect to a number of features compared with most other troodontids. Many of these differences are similar to conditions seen in avialans and dromaeosaurids^{5,17–19}. Laterally, the skull has an antorbital fossa that is much smaller than that of other non-avian theropods (except for oviraptorosaurians⁵) and a large orbit that is apparently confluent with the infra-temporal fenestra (Fig. 2a, b). No postorbital is preserved, but if present it was undoubtedly small, as in *Archaeopteryx*⁹. As in avialans¹⁹ and mononykines²⁰, there is no corresponding ascending postorbital process of the thin jugal to form a complete postorbital bar; however, there is a small dorsal expansion of the jugal at its posterior end (as in *Archaeopteryx*¹⁹), near where it contacts the quadrate, which is buttressed by an extremely small quadratojugal (Fig. 2a, b). The squamosal is also reduced and does not contact the quadrato-

jugal, and the quadrate has a sharp and ventrally located pterygoid ramus. The snout is very short (Fig. 2a, b). The premaxilla has a large pre-naris portion and a long ascending process that accounts for about one-half of the snout length. The anterior projection of the maxilla is very long, and shallow, and forms the entire ventral border of the extremely large external nares that extend posteriorly over one-half of the length of the maxillary tooth row. Consequently, the nasals are short and consistently wide along their entire length. Comparatively, the frontal is elongate, about 45% the total length of the skull, and is blunt with a slight transverse expansion anteriorly. A sagittal crest is absent on the parietal. The braincase is expanded dorsoventrally and transversely (much wider than the minimum interorbital region). The supraoccipital crest is prominent, divided by an unclosed suture, and is hourglass-shaped, bordering distinct epiotic/opisthotic ossifications. The teeth are unserrated and distally re-curved as in *Byronosaurus*¹⁴. As in dromaeosaurids, the posterior dorsal vertebrae bear distinct para-

pophyses; however, they are not as stalk-like as those in advanced dromaeosaurs (for example, *Velociraptor*). The radius is thin and proportionally much longer than in other troodontids (see Supplementary Information), about 95% of the humeral length. A prominent proximally projected process lies on the lateral margin of distal tarsal IV, a feature otherwise only seen *Microraptor gui*. As in dromaeosaurids¹⁸, pedal phalanges II-1 and II-2 both have a medially centred ventral heel.

The holotype preserves and clarifies several unknown features of troodontids. The cervicals are incipiently heterocoelous as in some dromaeosaurids. The first and second dorsals bear prominent ventral processes on the anterior half of the centra, which are distally ball-like. Two small fragments of flat bones may represent sternal rib fragments as found in other maniraptorans^{18,21}. A large furcula is present in loose articulation with the acromion process of the scapula. It is robust and flat in cross section, with an incipient hypocleidium and expanded distal articular ends (Fig. 2c), and is

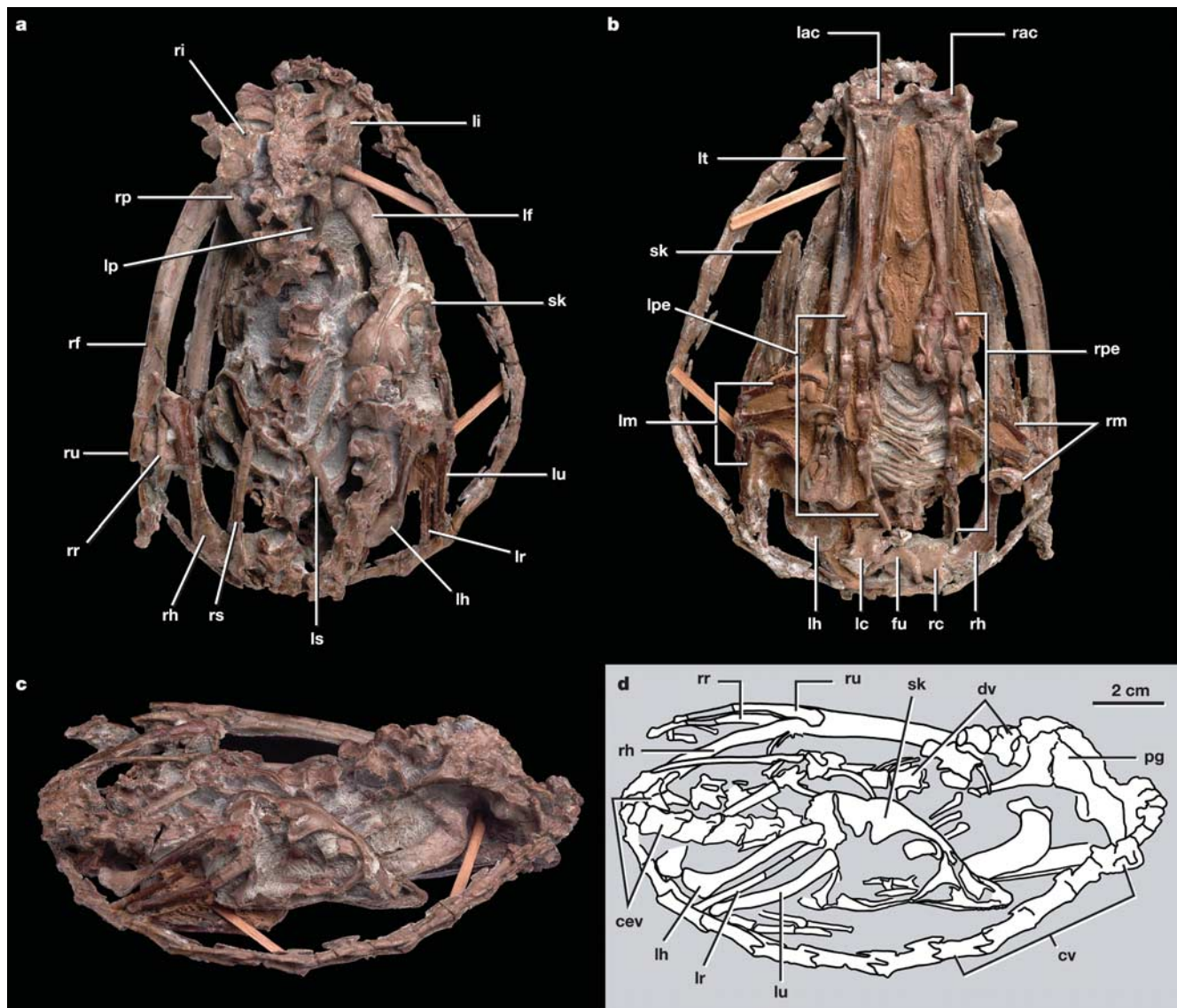


Figure 1 Holotype of *Mei long* (IVPP V12733). **a–c**, Photographs of the skeleton in dorsal (**a**), ventral (**b**) and dorsolateral (**c**) views. **d**, Line drawing of the skeleton in dorsolateral view. cev, cervical vertebrae; cv, caudal vertebrae; dv, dorsal vertebrae; fu, furcula; lac, left astragalus–calcaneum; lc, left coracoid; lf, left femur; lh, left humerus; li, left ilium; lm, left manus; lp, left pubis; lpe, left pes; lr, left radius; ls, left

scapula; lt, left tibia; lu, left ulna; pg, pelvic girdle; rac, right astragalus–calcaneum; rc, right coracoid; rf, right femur; rh, right humerus; ri, right ilium; rm, right manus; rp, right pubis; rpe, right pes; rr, right radius; rs, right scapula; ru, right ulna; sk, skull. Scale bar, 2 cm.

more 'U'-shaped and robust as in avialans rather than in dromaeosaurids^{5,17}. As in dromaeosaurids and avialans, the scapulocoracoid is 'L'-shaped and the scapula is positioned close to the neural spines of the dorsals and is almost parallel to the vertebral column. The coracoid has a large coracoid tubercle, a sub-glenoid fossa and is divided into an anterodorsal and an anteroventral surface by a low

ridge, as in many other maniraptorans⁵. The humerus projects laterally and the forearm and the manus fold in an avian mode (Fig. 1a, b), as in other maniraptorans⁵.

Inclusion of *M. long* into a recent phylogenetic analysis²² places *M. long* as the sister group to *Sinovenator* at the base of troodontids, and supports a monophyletic Deinonychosauria, which in turn is the sister group to Avialae (see Supplementary Information). For the included taxa this consensus solution is identical to that proposed by ref. 22.

Several studies have suggested that small size is crucial to the origin of flight and that miniaturization was responsible for many of the unique morphologies seen in avialans^{6,16,17}; thus, *M. long* provides further evidence for this theory. For instance, *M. long* and *Archaeopteryx* lack a jugal–postorbital and a quadratojugal–squamosal contact^{9,19}, and so might basal dromaeosaurids^{5,19}. The nasal–frontal articulation is weak in *M. long* and possibly also in basal dromaeosaurs. These features suggest that some kind of cranial kinesis (probably prokinesis) evolved in the early evolution of eumaniraptorans. Notably, these features are also found in mononykines²⁰. Derived larger members of the two deinonychosaurian groups often have the primitive states of these characters^{5,23} and show a non-avian akinetic skull.

This specimen displays the earliest recorded occurrence of the stereotypical sleeping or resting behaviour found in living birds. The body sits on folded symmetrical hindlimbs. The forelimbs are also symmetrical and extend laterally, folded avian-like next to the body, with the elbows slightly displaced laterally relative to the trunk (the left elbow more than the right, with the distal-most manus underneath the knees). The neck curves posteriorly on the left side of the body so that part of the head lies medial to the left elbow at the side of the trunk. This posture is identical to the stereotypical 'tuck-in' sleeping posture of many living birds. In the *M. long* holotype, we propose that this posture represents a life posture for the animal captured when it was sleeping or resting on the ground, based on the sedimentary context and comparative pose data of most other dinosaur specimens (see Methods).

Living tetrapods display diverse sleeping and resting postures, but only avians and some mammals rest on their folded limbs. Avians, owing to their long and very flexible necks, differ from mammals in tucking their heads between one of their anterior appendages and their torso. The *M. long* holotype is preserved in a remarkable life pose, capturing this tuck-in sleeping (or resting) posture. In birds, the tuck-in posture reduces surface area and conserves heat in the head, a major region of heat loss in these animals^{24,25}. It is therefore usually associated with heat conservation^{24,25}. From the phylogenetic position of the fossil we can tell that the tuck-in behaviour originated in the non-avian precursors to modern birds. Although we are unable to measure it directly, the physiological/thermal implications of this and other fossil evidence (for example, brooding and feathers) are highly suggestive that these animals shared a homeothermic physiology with modern avians. □

Methods

Life posture inference

The holotype of *M. long* is inferred to be buried in deposits when the animal was sleeping or resting, and thus preserves a life posture. Exceptionally well-preserved vertebrate specimens have been collected from the Lujiatun beds over the last few years, and it has been proposed that some of them provide information for dinosaur behaviour³. The Lujiatun beds are alluvial deposits mainly comprising tuffaceous conglomerate debris flows, sandstones and mudstones²⁶. Some of the most fossiliferous locations are considered to be the result of instant catastrophic mass mortality events^{26,27} preserved in tuffaceous ashes up to 3 m in thickness. Although not all are volcanic in origin, such Pompeii-like depositional conditions are present in other localities that preserve behavioural characters in fossil vertebrates^{1,28}. Other dinosaur specimens from the lacustrine beds of the Yixian formation²⁶ and specimens from the more classically fluvial lower sandstone beds (such as *Shenzhousaurus*²⁹) are not preserved in life posture, but in more typical death poses with heads bent back above the spine and the hind appendages and tails extended³⁰. Both the specimen itself and the sedimentology of the deposits from which this specimen was collected indicate that the preserved posture represents a life posture for the animal. Such a

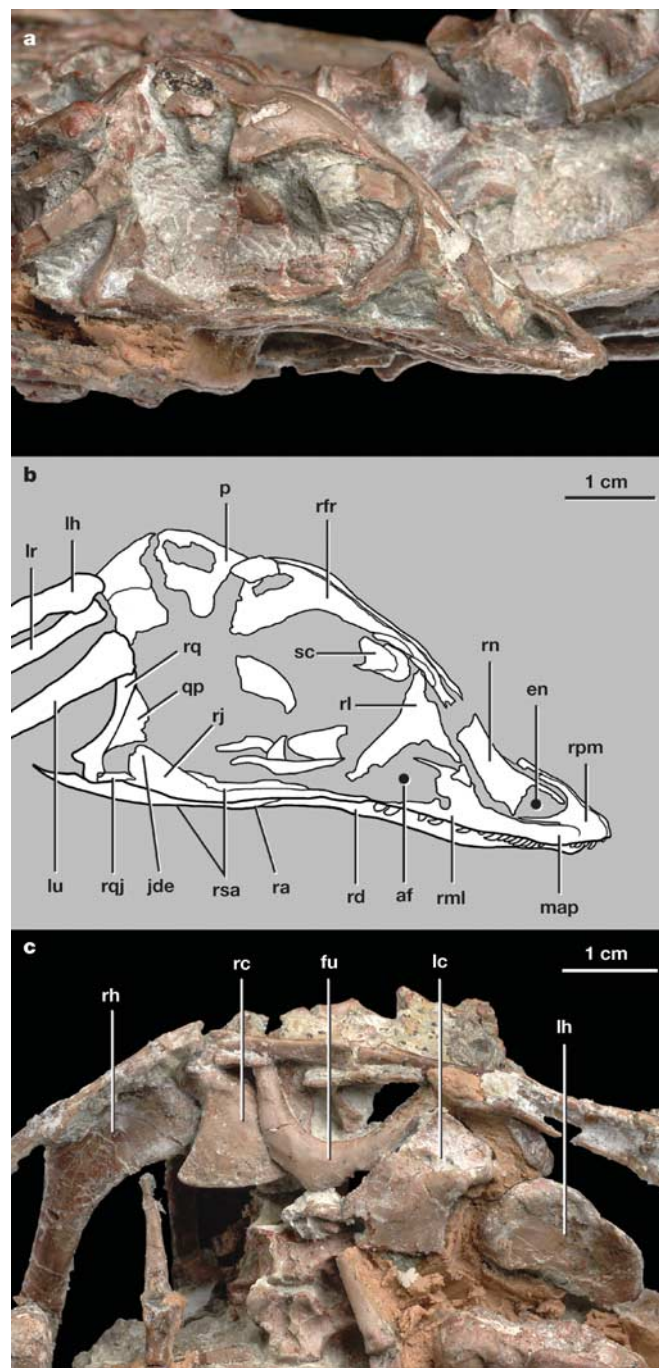


Figure 2 Holotype of *Mei long* (IVPP V12733). **a, b**, Photograph (**a**) and line drawing (**b**) of skull in right lateral view. Note that the right external naris is much smaller than its actual size due to the anterior displacement of the right nasal. **c**, Furcula in anteroventral view. af, antorbital fossa; en, external naris; jde, dorsal extension of posterior jugal; map, anterior projection of the maxilla; p, parietal; qp, pterygoid process of quadrate; ra, right angular; rd, right dentary; rfr, right frontal; rj, right jugal; rl, right lacrimal; rml, right maxilla; rn, right nasal; rpm, right premaxilla; rq, right quadrate; rqj, right quadratojugal; rsa, right surangular; sc, sclerotic plates. Scale bar, 1 cm.

posture can also be inferred from the Early Cretaceous troodontid *Sinornithoides* from the Ordos basin and perhaps for the nesting oviraptorid *Citipati* (IGM 100/979); however, these specimens are incomplete and more distorted^{1,11}.

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Adaptation varies through space and time in a coevolving host–parasitoid interaction

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One of the central challenges of evolutionary biology is to understand how coevolution organizes biodiversity over complex geographic landscapes. Most species are collections of genetically differentiated populations, and these populations have the potential to become adapted to their local environments in different ways. The geographic mosaic theory of coevolution incorporates this idea by proposing that spatial variation in natural selection and gene flow across a landscape can shape local coevolutionary dynamics^{1–7}. These effects may be particularly strong when populations differ across productivity gradients, where gene flow will often be asymmetric among populations⁸. Conclusive empirical tests of this theory have been particularly difficult to perform because they require knowledge of patterns of gene flow, historical population relationships and local selection pressures². We have tested these predictions empirically using a model community of bacteria and bacteriophage (viral parasitoids of bacteria). We show that gene flow across a spatially structured landscape alters coevolution of parasitoids and their hosts and that the resulting patterns of adaptation can fluctuate in both space and time.

Bacteria and bacteriophage have been used as model organisms for ecological and evolutionary studies since at least the 1960s and have proven particularly useful for studies of antagonistic coevolution^{9–13}. The coevolutionary dynamics of the bacterium *Escherichia coli* and the bacteriophage T7 are well studied, and can be divided into a series of phenotypic classes based on patterns of resistance and counter-resistance². Ancestral, T7-sensitive bacteria (B_0) evolve resistance to ancestral bacteriophage (T_7), after which they are referred to as first order resistant bacteria (B_1). This change most often occurs through mutations that change or eliminate the cell surface receptor molecule. T7 can then evolve to attack the first order resistant bacteria and are referred to as host-range mutants (T_7). Most often this occurs through mutations that change the T7 tail fibres, resulting in an expanded host range (that is, T_7 are able to attack both B_0 and B_1). Second order resistant bacteria (B_2) that are resistant to T_7 and T_7 can also arise and eventually invade the community. T7 have not yet been observed to evolve the ability to infect B_2 (ref. 9). Because T7 directly kills the bacteria that it infects and also the generation time of T7 is shorter than that of its host, T7 can be thought of as a parasitoid rather than a parasite or predator.

We evaluated how dispersal across a spatially structured landscape shapes the coevolutionary dynamics of *E. coli* and T7 by establishing a stepping-stone, source–sink experimental landscape of microbial communities. We directly manipulated dispersal of T7 and *E. coli* along a resource (productivity) gradient in two landscapes (Fig. 1). In one landscape, continuous culture devices (chemostats) containing T7 and *E. coli* were linked by dispersal in a stepping-stone fashion. Every two days, we dispersed 10% of the community from the highly productive source community to a community with an intermediate level of resources, and then 10% of that community to a low productivity (sink) community. In the

second landscape, chemostats also varied in resource availability (high, intermediate and low productivity) but were not linked by dispersal. Each landscape was replicated two to three times.

We predicted that local adaptation (measured as the infectivity of bacteriophage on sympatric versus allopatric hosts) would differ across the experimental landscape as a result of a geographic selection mosaic driven by resource variability. Selection mosaics occur when natural selection on interactions varies among communities owing to differences in the biotic or abiotic environment^{1,14}. On the basis of past research in similar systems, we predicted that experimentally-imposed differences in resource availability should drive variation in the abundance of B_1 across the landscapes, which should then influence the evolution of the bacteriophage populations¹⁵. The evolution and invasion of resistant bacteria is often faster in highly productive communities¹⁵, therefore the abundance of B_1 should be greater in the high productivity communities than in the low productivity communities. In addition, T_{71} should dominate the high productivity communities earlier than the low productivity communities. These differences in the abundance of B_1 and the timing of invasion of T_{71} , driven by differences in productivity, provide evidence for a geographic selection mosaic.

Twenty-four hours after the experiment was initiated, the abundance of B_1 (which evolved *de novo* in each community) was approximately five times greater in the highly productive source communities than in each of the other two communities, and was slightly greater in the intermediate productivity community than in the low productivity community (analysis of variance among groups (ANOVA): $F_{2,10} = 21.09$, $P < 0.001$). In addition, T_{71} appeared in the highly productive source communities first (on day 5). T_{71} invaded the intermediate productivity communities approximately two days later and did not appear in the low productivity communities until approximately day 11. Thus, a selection mosaic was present across our experimental landscape (Fig. 1).

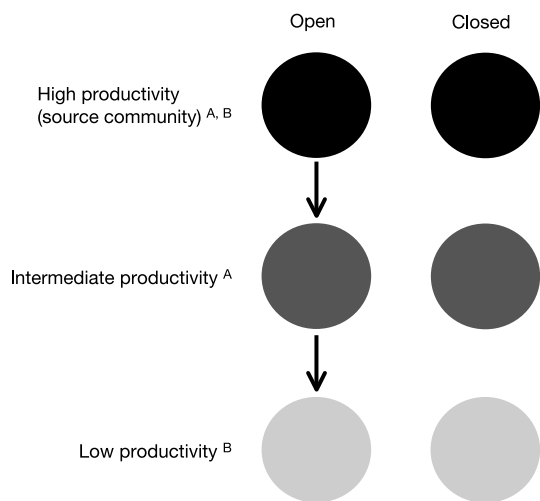


Figure 1 The experimental landscapes. Circles represent communities with different levels of productivity that are either open (indicated by arrows) or closed to dispersal. Greyscale represents the intensity of selection (dark = most intense, light = least intense) as indicated by the abundance of B_1 on day 1 and the first date of invasion of T_{71} . The abundance of B_1 was greatest in the high productivity communities and T_{71} invaded these communities earliest. (*Post hoc* planned comparisons of B_1 abundance among communities. Significant differences indicated by A and B; A: $F_{1,10} = 27.32$, $P < 0.001$; B: $F_{1,10} = 33.35$, $P < 0.001$).

We next asked how gene flow influenced patterns of local adaptation through space and time. Under relatively high levels of gene flow, local adaptation may not be evident because gene flow can act as a homogenizing force, preventing independent evolution in local populations. In contrast, at lower levels, gene flow may act as a source of beneficial mutations to local populations and therefore increase levels of local adaptation^{16–19}. Dispersal (gene flow) occurred at a low rate in our experiment. For the bacteriophage populations, the average fraction of immigrants (m)²⁰ was 0.002 per generation into the intermediate productivity community and 0.005 per generation into the low productivity community (values were lower for the bacterial populations). Thus, we predicted that dispersal from higher productivity to lower productivity communities would increase levels of local adaptation in the lower productivity communities, relative to the communities that were closed to dispersal, owing to the influx of genetic variation.

We also predicted that, although dispersal would lead to increased levels of local adaptation, it would also result in increased variation in adaptation through time^{16,17,21}. This is predicted to occur because at a given point in time, gene flow can enhance (through increased genetic variation) or reduce (through dilution of beneficial alleles) local adaptation, although at relatively low levels it is predicted to have an overall positive effect when averaged over space and time. Whether gene flow has a net positive or negative effect at a given point in time and space is random; thus variation in local adaptation should be greater than in the closed communities.

We used local adaptation in the bacteriophage as an indicator of ongoing coevolution in the bacteria and bacteriophage populations through time. Local adaptation in the bacteriophage reflected combined changes in the host and parasitoid as they coevolved through time. In other words, local adaptation in the bacteriophage population at one time point should result from changes in the bacteriophage that enhance its ability to attack the bacteria, whereas local maladaptation in the bacteriophage at a subsequent point in time should reflect the evolution of resistance in the bacteria. This cycle should continue through time, resulting in variation in levels of adaptation owing to the coevolutionary arms race between the bacteriophage and bacteria (although under some circumstances it is possible for local adaptation to consistently improve with time²²).

We measured local adaptation of the bacteriophage through time to contemporary B_1 (that is, isolated from the same time point as the bacteriophage). This allowed us to assess co-adaptation of the bacteriophage and bacteria as both evolved through time. We considered the bacteriophage to be locally adapted if their infectivity was greater when tested on the numerically dominant sympatric host (from the same community) than on the dominant allopatric host (from a different community, but at the same productivity level; see Methods).

We first analysed patterns of local adaptation in the bacteriophage populations from the source (high productivity) communities. This provided us with a baseline understanding of the dynamics of adaptation, as well as information on the potential causes of coevolutionary dynamics in the intermediate and low productivity regions that were linked to the source community. Patterns of local adaptation through time were not significantly different in the open source communities compared to the closed communities (ANOVA, time $\times$ dispersal: $F_{1,4} = 6.747$, $P = 0.06$).

Table 1 Results from the repeated measures ANOVA (Pillai Trace test statistic)

	Hypothesis d.f.	Error d.f.	<i>F</i>	<i>P</i>
Time	2	3	8.387	0.06
Time $\times$ productivity	4	8	5.408	0.021
Time $\times$ dispersal	2	3	24.187	0.014
Time $\times$ dispersal $\times$ productivity	4	8	5.082	0.025

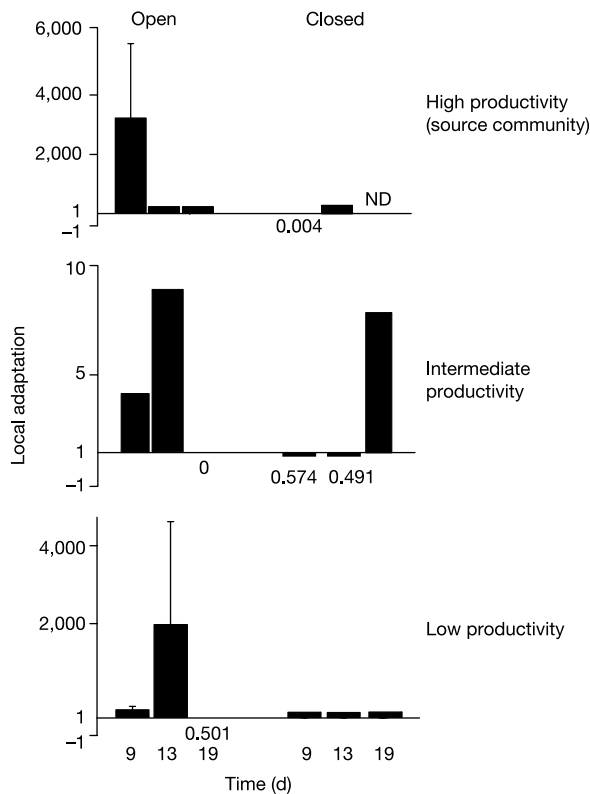


Figure 2 Adaptation in the bacteriophage populations from each productivity level. Bacteriophage populations measured with (open) and without (closed) dispersal on days 9, 13 and 19 of the experiment ($\pm$ standard error). Values above one indicate local adaptation. Values below one indicate maladaptation. Values equal to one indicate no evidence of adaptation or maladaptation. ND = no data for the high productivity community on day 19, where samples were lost due to contamination. Where values are small they are written below the columns.

This was as we expected—the only difference between the open and closed source communities was emigration every two days out of the open communities. This rate of emigration increased the rate of loss (and thus growth rate) of the bacteriophage population in the source communities by only 1%, not enough to significantly alter the rate of evolution during the course of this experiment.

When local adaptation was analysed for all communities for three time points during the experiment, we found that dispersal across the selection mosaic strongly influenced patterns of adaptation, and that these patterns changed through time and with differences in productivity (Table 1; Fig. 2). Measures of adaptation were greatest in the open/high productivity communities on day 9 and appeared lagged in time in the open/intermediate and open/low productivity communities. Local adaptation was lower in the closed communities than in the open communities. In addition, there was more evidence for maladaptation in the closed communities compared to the open communities. Overall, these results suggest that gene flow was acting as a source of beneficial mutations in the open communities, which drove the dynamics of adaptation. Similar results have been found in other studies of bacteriophage–bacteria coevolution on much smaller spatio-temporal scales²³. In our experiment, dispersal from the highly productive source community was seeding the intermediate and low productivity communities with genetic variation, resulting in time-lagged patterns of adaptation in these communities.

In addition to evaluating mean values of adaptation at a series of time points during the experiment, we also assessed how dispersal across a heterogeneous landscape influenced variation in adaptation of bacteriophage through time. Variation in adaptation was

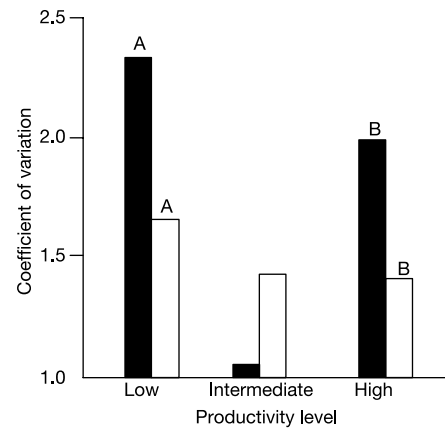


Figure 3 Variation of the adaptation ratio. The coefficient of variation of the adaptation ratio calculated from day 9, 13 and 19 for each of the open (filled bars) and closed (open bars) communities. Letters indicate significant differences (the same letter above a bar indicates that the values depicted by the bars are significantly different).

approximately 50% greater in bacteriophage from the open/high and open/low productivity communities than in the closed communities (Fig. 3), as predicted. We observed the opposite pattern in the intermediate productivity communities: variation in adaptation was greater on average in the closed than in the open communities. However, this pattern was driven solely by one of the two replicate runs of the experiment; the other run was consistent with the pattern observed for the other productivity levels. In general, our results suggest that although dispersal led to local adaptation in the bacteriophage, it also increased variation in adaptation through time.

Based on our results, it seems likely that the equivocal results of past work on the role of spatial heterogeneity in patterns of adaptation are due to the limited snapshot in time in which these measures were taken. Our results show that patterns of adaptation shaped by coevolution can be highly dynamic through space and time. Overall, our results provide the first direct empirical evidence that gene flow across a spatially structured landscape can alter the dynamics of coevolution. Our experimental design allowed us to evaluate both the separate and combined influences of time and gene flow across a heterogeneous landscape on coevolution. The results demonstrate that even in a relatively simple, controlled experimental setting, coevolutionary interactions are highly dynamic when embedded within a geographic mosaic. □

Methods

Experimental design

The experiment was conducted in continuous culture devices known as chemostats^{15,24}. The experiment ran for approximately 19 days (approximately 200 bacterial generations and 1,000 generations of bacteriophage), and, unless otherwise noted, under previously described conditions¹⁵. Nutrients entered the chemostats at a predetermined rate, which was equal to the rate at which unutilized resources, waste and organisms were removed.

Each chemostat represented a community with a specific level of productivity, determined by the input concentration of glucose, the growth limiting resource for bacteria. Three levels of productivity were used (high = 1 mg ml⁻¹, intermediate = 100 μ g ml⁻¹ or low = 10 μ g ml⁻¹ glucose input). The communities were either connected by dispersal in a stepping-stone design, which occurred from high to intermediate to low productivity, or closed to dispersal. Dispersal occurred through the manual removal of 10% of the community (bacteriophage and bacteria combined) every 48 h. Each landscape of six chemostats was replicated two to three times.

At the start of each run of the experiment, all chemostats were inoculated with the same ancestral strains of T7 bacteriophage (obtained from the American Type Culture Collection) and of *E. coli* B (strain REL 607; ref. 24). Chemostats were sampled one day after inoculation to verify that populations of bacteriophage and bacteria were present in all chemostats and then every 48 h (immediately before dispersal) throughout the duration of the experiment. The same volume of the community (approximately 5 ml) was removed from each chemostat for sampling. A portion of the 5 ml was used for dispersal in the open communities (3 ml). Another portion of the sample was used to determine population sizes of the bacteriophage and bacteria. The remainder of the sample was stored at -80 °C, for further detailed *post hoc* evaluation of patterns of adaptation.

Analyses

We determined the population size of the bacteriophage by adding 30 µl of chloroform to a 1 ml subsample of each chemostat (to remove bacteria) and plating 100 µl of the purified subsample of bacteriophage with 300 µl of an overnight culture of bacteria in 3 ml of soft agar. We counted the number of plaques (spots cleared of bacteria by the bacteriophage) after 24 h. Because the bacteriophage were well mixed before plating, each plaque was presumed to result from a single viral particle. A lawn of ancestral bacteria (B_0) was used to determine the total size of the bacteriophage population and a lawn of resistant bacteria (B_1) was used to calculate the number of host-range mutants (T_{71}) in the bacteriophage population.

We determined the population size of the bacteria by plating 100 µl of a subsample (without chloroform treatment) and counting the number of bacterial colonies present after 24 h of incubation. To determine the size of the B_1 population, we plated the bacteria with an equal volume of ancestral bacteriophage.

To investigate local adaptation in the bacteriophage through time, we isolated two to three colonies of B_1 from the same day (day 9, 13 and 19) from each treatment during each run of the experiment. For each adaptation assay, overnight cultures of the colonies were grown up in 1 mg ml⁻¹ glucose media. We plated replicate samples of T_{71} from each time point on a lawn of sympatric (from the same chemostat) and allopatric (from a different chemostat, but at the same productivity level) B_1 from the overnight culture. We used the 'efficiency of plating' (the number of plaques on each host) as a measure of bacteriophage infectivity.

We calculated adaptation as the ratio of the number of plaques formed by the numerically dominant bacteriophage on the dominant sympatric host to the number of plaques formed on the allopatric host. Ratios of the number of plaques formed on the bacterial isolates from each replicate run of the experiment were averaged to give a mean ratio for each run. Only one replicate run of the experiment was used in assays of the closed/high productivity community due to contamination. There were often no plaques on allopatric hosts, therefore we coded the data by adding one to both the numerator and the denominator to allow calculation of a ratio. A value above one indicates local adaptation and a number below one indicates local maladaptation (that is, the number of plaques was higher on the allopatric host than on the sympatric host). All ratios were log-transformed before analysis.

Variation in adaptation was assessed by calculating the coefficient of variation of the adaptation ratios. Significance was tested using an *F*-test, with a sequential Bonferroni correction for five pairwise comparisons²⁵. Inspecting the data in detail showed that the reversed pattern for the open and closed intermediate productivity communities was driven by one of the two replicate runs of the experiment. Thus, we did not include data from the intermediate productivity communities in the analyses.

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Hedgehog signalling controls eye degeneration in blind cavefish

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Hedgehog (Hh) proteins are responsible for critical signalling events during development¹ but their evolutionary roles remain to be determined. Here we show that *hh* gene expression at the embryonic midline controls eye degeneration in blind cavefish. We use the teleost *Astyanax mexicanus*, a single species with an eyed surface-dwelling form (surface fish) and many blind cave forms (cavefish)², to study the evolution of eye degeneration. Small eye primordia are formed during cavefish embryogenesis, which later arrest in development, degenerate and sink into the orbits. Eye degeneration is caused by apoptosis of the embryonic lens, and transplanting a surface fish embryonic lens into a cavefish optic cup can restore a complete eye^{3–5}. Here we show that *sonic hedgehog* (*shh*) and *tiggy-winkle hedgehog* (*twhh*) gene expression is expanded along the anterior embryonic midline in several different cavefish populations. The expansion of *hh* signalling results in hyperactivation of downstream genes, lens apoptosis and arrested eye growth and development. These features can be mimicked in surface fish by *twhh* and/or *shh* overexpression, supporting the role of *hh* signalling in the evolution of cavefish eye regression.

Cave animals often lose their eyesight during evolution in perpetual darkness (Fig. 1a, b), but the evolutionary and developmental mechanisms underlying these dramatic phenotypes are unknown⁶. Over the last 10,000 yr, at least four different *Astyanax* cavefish populations may have evolved various degrees of eye degeneration independently^{7–10}. In addition to blindness, cavefish show other regressive and constructive morphological features, including loss of melanin pigment, modifications in the orbital skeleton and increases in jaw size, maxillary teeth and taste buds^{2,10}. We previously reported that *pax6* expression is downregulated in the presumptive optic fields in several cavefish populations, suggesting that upstream regulators of *pax6* may control eye degeneration¹¹.

To detect early changes in eye development, optic morphogenesis and gene expression patterns were compared in cavefish and surface fish embryos. The results showed that the optic vesicle is reduced in size (Fig. 1c, d) and the ventral sector of the optic cup is lost (Fig. 1e, f)

in cavefish embryos. Vertebrate optic primordia are patterned by reciprocal transcriptional repression between the transcription factors Pax2, which is expressed in the optic stalk, and Pax6, which is expressed in the optic cup^{12,13}. *In situ* hybridization showed that *pax2a* expression is expanded in the cavefish optic vesicle and optic cup (Fig. 1c, d, g, h). Changes in the optic primordium were confirmed by examining *vax1*, which is also expressed in the optic stalk^{14,15}. Similar to *pax2a*, *vax1* expression was expanded in the cavefish optic cup (Fig. 1i, j), suggesting that upregulation of these genes modifies the eye primordia.

Hh proteins emanating from the anterior embryonic midline control the expression of *pax2*, *vax1* and *pax6* in vertebrate optic primordia^{13–17}. Comparisons of *hh* gene expression in surface fish and cavefish embryos revealed several highly reproducible changes along the anterior midline. At the neural plate stage, *shh* and *twhh* expression was expanded in the cavefish prechordal plate relative to non-overlapping *pax2a* expression at the future midbrain–hind-brain boundary¹⁸ and *dlx3b* expression at the border of the neural plate¹⁹ (Fig. 2a–d, i–j). The cavefish *shh*-expressing domain is about ten cells wide at its greatest lateral extent, whereas it is only about six cells wide in surface fish ($n = 6$). During optic vesicle formation, *shh* expression extended further anterior and dorsal in cavefish, curling around the rostrum (Fig. 2e–h). To confirm *hh* expansion, we also examined the expression patterns of *hh* downstream target genes. The results showed that *ptc2* (Fig. 2k, l), which encodes a

shh receptor^{20,21}, and *nkx2.1a* (Fig. 2m, n), which encodes an *hh*-regulated transcription factor²², were also expanded along the cavefish midline. The significance of *hh* expansion in eye loss was established in two ways. First, two additional cavefish populations

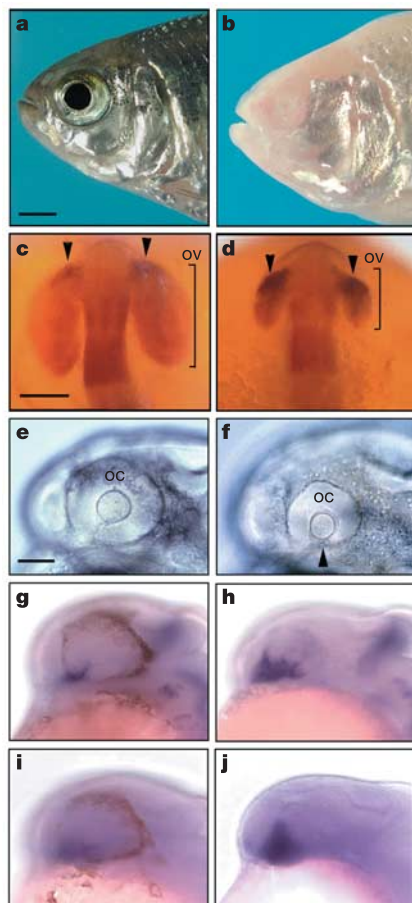


Figure 1 Changes in surface fish (left column) and cavefish (right column) eyes and optic primordia. **a, b**, Adults. **c, d**, *pax2a* expression (arrowheads) in optic vesicles (ov). **e, f**, Optic cup (oc) morphology showing loss of ventral sector (arrowhead) in cavefish. **g–j**, *pax2a* (**g, h**) and *vax1* (**i, j**) expression in optic vesicles. Shown are dorsal (**c, d**) and lateral (**a, b, e–j**) views with anterior on left. Scale bars: 0.5 cm (**a**), 250 μ m (**c**) and 150 μ m (**e**); same magnification in **a** and **b**, **c** and **d** and **e–j**.

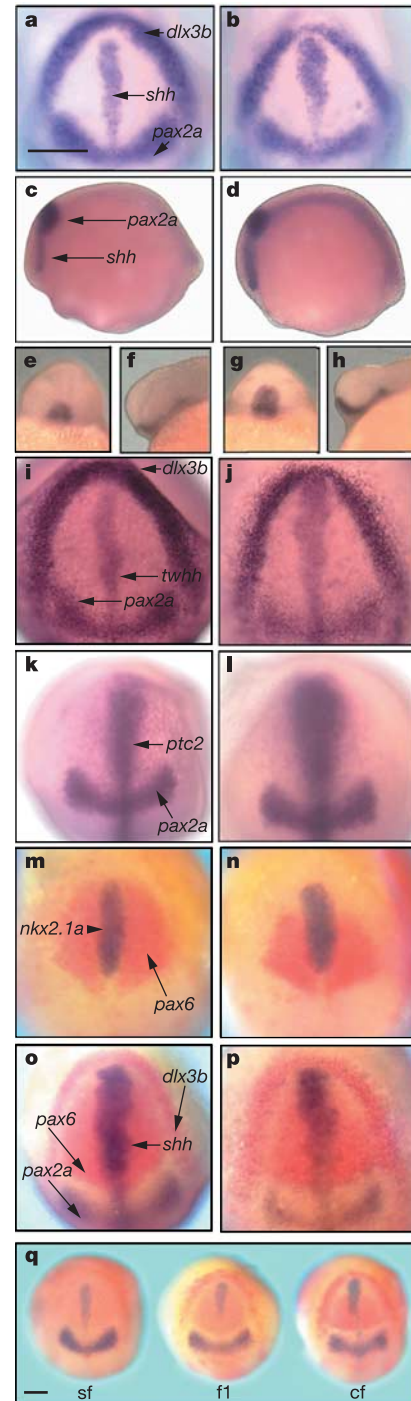


Figure 2 *hh* and *hh*-related gene expression in fish embryos. (Surface fish: **a, c, e, f, i, k, m**; cavefish: **b, d, g, h, j, l, n–p**) **a–j**, *shh* (**a–h**) and *twhh* (**i, j**) expression relative to *dlx3b* and/or *pax2a* expression in early tailbud (**a–d, i, j**) and 10-somite (**e–h**) embryos. **k–n**, *ptc2* (**k, l**) and *nkx2.1a* (**m, n**) expression relative to *pax2a* and *pax6* expression respectively in early tailbud embryos. **o–q**, *shh* (blue) expression relative to *dlx3b* (red), *pax2a* (blue) and *pax6* (red) expression in early tailbud-stage Chica (**o**) and Los Sabinos (**p**) cavefish embryos and surface fish (sf), F1 (f1) and cavefish (cf) embryos (**q**). Shown are dorsal (**a, b, i–q**), lateral with anterior on left (**c, d, f, h**) and rostral (**e, g**) views. Scale bars, 250 μ m; same magnification in **a–p**.

were examined and these also exhibited expansion of *shh* expression at the embryonic midline (Fig. 2o, p). Second, *shh* expression was examined in the progeny of a cross between surface fish and cavefish. The F1 embryos, which have uniformly small eyes relative to surface fish^{2,23}, showed a midline *shh* domain intermediate in width between surface fish and cavefish (Fig. 2q).

To test the possibility that eye degeneration is caused by increased Hh signalling, we overexpressed *hh* by injecting *twhh* and/or *shh* messenger RNA into surface fish embryos¹⁶. As a result, *shh* and *nkx2.1a* expression were expanded at the anterior midline and *pax6* expression was contracted in the presumptive optic regions of the injected embryos (Fig. 3a–c; data not shown). Subsequently, smaller optic vesicles (Fig. 3f) with expanded *pax2a* (data not shown) and *vax1* (Fig. 3d, e) expression and ventrally reduced optic cups

(Fig. 3g) were observed. The affected features usually occurred unilaterally, probably because mRNA was injected on one side of the bilateral embryonic axis. However, the unilateral changes were observed in a proportion of embryos that paralleled those in which *shh* was expanded on only one side of the anterior midline. By the larval stage, 78% ($n = 50$) of these embryos exhibited a ventrally diminished retina and a small or undetectable lens (Fig. 3h). Adults that developed from these embryos were missing an eye (Fig. 3i) and did not respond to light focused on the affected orbit. *Shh* overexpression had no detectable effects on body pigmentation, the orbital skeleton, jaw width or maxillary tooth number, although there was a small increase in taste bud number on the lips (6%, $n = 30$). Similar effects were observed following injection of *shh/twhh* or *shh* mRNA. The results indicate that *hh* overexpression can phenocopy cavefish eye degeneration.

To determine the effects of reducing Hh activity, cavefish embryos were treated with cyclopamine, an inhibitor of the Hh signalling pathway²⁴. Cyclopamine-treated embryos showed larger optic vesicles with diminished *pax2a* (data not shown) and *vax1* domains (Fig. 3j, k). Later in development, the lens was 30% larger than in controls ($n = 38$), supporting a partial restoration of eye development. However, eye development was not completely restored in cyclopamine-treated cavefish embryos, probably due to the late requirement for *shh* and *twhh* expression in the developing retina^{25,26}.

Further experiments were conducted to determine the effects of increased midline signalling on lens and eye development. First, 43% ($n = 42$) of the *shh* mRNA-injected embryos assayed by TdT-mediated dUTP nick end labelling assay (TUNEL)³ exhibited apoptosis in one or both lens vesicles (Fig. 4a, b). Second, eye development was arrested in 36% ($n = 25$) of the cases in which a

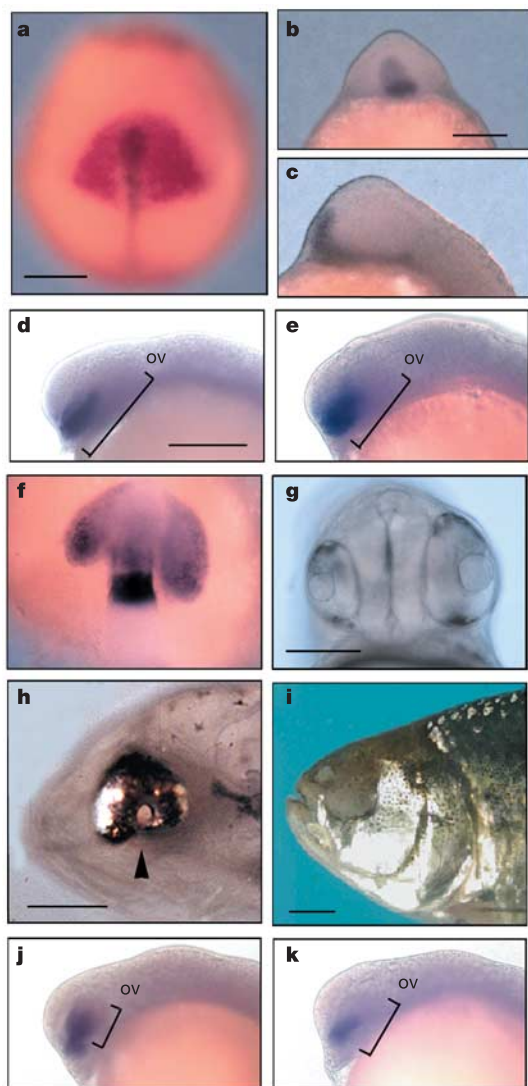


Figure 3 *hh* control of eye formation. **a–i**, Effects of *twhh* and *shh* (**a–c**, **f–i**) or *shh* (**e**) mRNA injection in surface fish embryos. **a–c**, Early tailbud (**a**) and 10-somite (**b**, **c**) embryos with expanded *shh* (blue) and asymmetric *pax6* expression (**a**, red). **d**, **e**, Expanded *vax1* expression in the optic vesicle (ov) of an *hh* mRNA-injected embryo (**e**) compared to a control (**d**). **f**, **g**, Unilateral reduction of optic vesicle (**f**, *pax6* staining) at the 18-somite stage and optic cup (**g**) at the hatching stage in *hh* mRNA-injected embryos. **h**, **i**, Ventral eye reduction (**h**, arrowhead) and eye loss in a blind cavefish phenocopy (**i**) after *hh* mRNA injection. **j**, **k**, *vax1* expression in cyclopamine-treated (**k**) and control (**j**) cavefish embryos. Shown are dorsal (**a**, **f**), lateral with anterior on left (**c–e**, **h–k**) and rostral (**b**, **g**) views. Scale bars: 200 μ m (**a**), 250 μ m (**b**, **d**), 150 μ m (**g**), 100 μ m (**h**) and 0.5 cm (**i**); same magnification in **b** and **c**, **d–f**, and **j** and **k**.

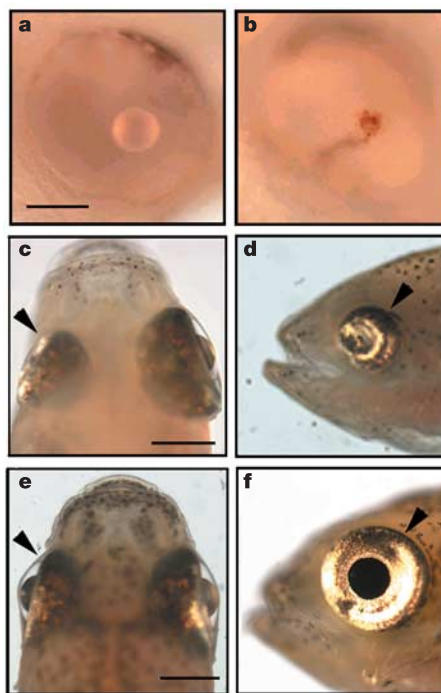


Figure 4 Effects of *shh* overexpression on surface fish lens development. **a**, **b**, TUNEL-positive apoptotic lens (red) in a *shh* mRNA-injected embryo (**b**) but not in a control (**a**). **c**, **d**, Inhibition of eye growth after lens transplantation from *shh* mRNA-injected embryo into the optic cup of a normal embryo. **e**, **f**, Restoration of eye growth after transplantation of a lens from a normal embryo into the affected optic cup of a *shh* mRNA-injected embryo. Arrowheads: transplantation side. Shown are dorsal (**c**, **e**) and lateral (**a**, **b**, **d**, **f**) views. Scale bars: 150 μ m (**a**) and 100 μ m (**c**, **e**); same magnification in **a** and **b**, **c** and **d**, and **e** and **f**.

lens was transplanted from a *shh* mRNA-injected surface fish embryo into the optic cup of a normal surface fish embryo (Fig. 4c, d). In contrast, eye development was not affected by lens transplantation from a normal surface fish embryo into the optic cup of another normal surface fish embryo (data not shown, $n = 118$). Third, eye development was partially or completely restored in 35% of the cases ($n = 17$) in which a normal lens was transplanted into the arrested eye of a *shh* mRNA-injected surface fish embryo (Fig. 4e, f). The results suggest that *shh* overexpression inhibits eye growth and development by inducing lens apoptosis.

We conclude that eye degeneration is mediated by expanded midline signalling in cavefish embryos. Accordingly, a small increase in Hh signalling at the embryonic midline is amplified by the induction of lens apoptosis to elicit a large negative effect on eye growth and development. Thus, *shh* and *twhh* could represent two of the four to six genes estimated to be involved in cavefish eye loss²³, although it is possible that upstream regulatory genes rather than *hh* genes themselves may be mutated during cavefish evolution. The latter hypothesis would explain coordinated changes in expression of the two *hh* paralogues.

Eye regression in cave animals has been attributed to loss-of-function mutations in eye genes, which may accumulate without penalty under conditions of relaxed selection for eyesight^{6,23}. The control of eye degeneration by a gain of function in *hh* or related midline-signalling genes in *Astyanax* cavefish raises the alternative possibility that eye regression could be driven by natural selection for an adaptive trait(s). Understanding the adaptive significance of enhanced Hh signalling is expected to provide key insights into the mechanisms controlling the evolution of blind cavefish. □

Methods

Biological materials and procedures

A. mexicanus embryos were obtained as described previously^{3–5,10,11}. All experiments were conducted with Pachón cavefish unless otherwise indicated.

Probes and *in situ* hybridization

Antisense RNA probes were generated from complete surface fish *shh* and *pax6* complementary DNAs, a partial *twhh* cDNA and *pax2a*, *dlx3b*, *vax1*, *ptc2* and *nkx2.1a* DNAs amplified from surface fish RNA by polymerase chain reaction (PCR) with reverse transcription. The *twhh* probe, which contains the 5' UTR and part of the amino-terminal coding sequence of the mRNA, showed a different expression pattern than *shh* in developing fin buds and is therefore considered to be gene specific. The following oligo primers were used in PCR reactions: *pax2a* CAGCCTTCCATCTATCTCCAG (forward) and CCGTAAACTCTCCACTACCCC (reverse), *dlx3b* GCCGGGATCCAARGAYTCN CCNACNYTNCC (forward) and GCCGGAATTCGARTTRCANGCCATNGARTC (reverse), *vax1* GCTCCATMCGRGARARATCAT (forward) and TTYTTCCTGCCTTT CCT (reverse), *ptc2* GTGTCTMTKTATGGAAAATCTTGG (forward) and TGACCTTACTCTSTTTCGG (reverse) and *nkx2.1a* CTSCRCBCYTACCARGASRS (forward) and GCSGASAGGTAYTTTGYTG (reverse). Two-colour *in situ* hybridizations¹¹ were done using digoxigenin-(blue) and fluorescein-(red) labelled RNA probes with the colour developed using NBT/BCIP or Fast Red respectively.

hh up- and downregulation

Constructs to produce mRNAs were made by inserting the full-length surface fish *shh* cDNA into the pSP64T plasmid. In some experiments we injected zebrafish *shh* and *twhh* mRNAs (1:1) made from pT7TS plasmids. The blastomeres of embryos at the 1–4-cell stage were injected with 0.4 ng ml⁻¹ (2 nl total volume) *hh* mRNA or control green fluorescent protein mRNA. Embryos were treated with 20–100 μM cyclopamine (5 mM stock solution in 95% ethanol; Toronto Research Chemicals) from 30% epiboly to hatching.

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Correspondence and requests for materials should be addressed to W.R.J. (jeffery@umd.edu). The *Astyanax* surface fish *pax6*, *shh*, *dlx3b*, *twhh*, *ptc2*, *nkx2.1a*, *pax2a* and *vax1* DNA sequences have been deposited in the GenBank database under accession numbers AY651762, AY661431, AY661432, AY661433, AY661434, AY661435, AY661436 and AY661437, respectively.

A relative signalling model for the formation of a topographic neural map

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The highly ordered wiring of retinal ganglion cell (RGC) neurons in the eye to their synaptic targets in the superior colliculus of the midbrain has long served as the dominant experimental system for the analysis of topographic neural maps^{1–3}. Here we describe a quantitative model for the development of one arm of this map—the wiring of the nasal–temporal axis of the retina to the caudal–rostral axis of the superior colliculus. The model is based on

RGC–RGC competition that is governed by comparisons of EphA receptor signalling intensity, which are made using ratios of, rather than absolute differences in, EphA signalling between RGCs⁴. Molecular genetic experiments, exploiting a combinatorial series of EphA receptor knock-in and knockout mice, confirm the salient predictions of the model, and show that it both describes and predicts topographic mapping.

During development, nervous systems are organized into precise representations of the external world¹. These frequently take the form of a topographic map—an ordering of axonal connections in which the positional coordinates of a set of input neurons are mapped to the corresponding coordinates of their targets. The model for such maps is the projection that connects the RGCs of the eye to their targets in the superior colliculus (SC; referred to as the tectum in non-mammalian vertebrates)^{1–3,5}. In this projection, the nasal–temporal (NT) and dorsal–ventral axes of the retina are mapped to the caudal–rostral and lateral–medial axes, respectively, of the SC (Fig. 1a).

The chemoaffinity hypothesis posits that axonal connections within a topographic map may be specified by interacting molecular tags that are distributed in complementary gradients on both projecting axons and their targets². In the retinocollicular map, members of the EphA family of receptor tyrosine kinases, together with their ephrin-A ligands, are thought to function as these graded tags^{5–7}. EphA5 and EphA6 are expressed in a low-nasal-to-high-temporal gradient in the mouse retina^{4,8}, and ephrin-A2 and ephrin-A5 are expressed in a low-rostral-to-high-caudal gradient in the SC^{8,9} (Fig. 1a). EphA4 is also expressed by mouse RGCs but is ungraded^{4,10–12}. Thus, retinal neurons with the most EphA receptors connect to collicular targets that carry the fewest ephrin-As, and vice versa (Fig. 1a). This is consistent with the demonstration that the ephrin-As are potent chemorepellents for RGC axons, most notably for those that originate in the temporal retina^{10,13,14}.

We have previously described a set of knock-in mice that permit a quantitative assessment of the role of EphA receptors in retinocollicular mapping⁴. In these mice, the *Isl2* (*Isl2*) gene, which is expressed at a constant level in a subset of RGCs randomly scattered across the retina, was used to drive ectopic expression of the EphA3 receptor⁴. EphA3 is not normally expressed in mouse RGCs^{4,8} but binds ephrin-A2 and ephrin-A5 with high affinity^{6,12}. The retinocollicular maps that develop in the *Isl2-EphA3* mice suggest that RGCs compete for target sites in the SC through relative, and not absolute, difference comparisons in EphA receptor signalling intensity⁴. If this is indeed so, then the apparent linearity of the mouse retinocollicular map⁴ requires that C_v , the concentration per RGC of the two receptors that are graded (EphA5+EphA6), must vary, as a function of position along the retinal axis, in proportion to itself (see Box 1, equation (1)); that is, that the retinal gradient for these receptors must be an exponential¹⁵ (equation (2)). Because C , the summed concentration of all EphA receptors per RGC (ΣEphA), is equal to $C_v + C_4$ (where C_4 is the constant concentration of EphA4), the full EphA gradient should be described by an exponential plus a constant (equation (3)).

To determine whether this is the case, we quantified *EphA4*, *EphA5* and *EphA6* messenger RNA levels in RGCs. (These seem to be the only relevant EphAs in the mouse^{4,16}.) We performed this quantification across the NT axis of the retina at postnatal day 1 (P1), a time at which RGC competition in the SC is underway¹⁷ (see Methods). We measured mRNAs because the analysis below requires firm numbers for the expression level per RGC of each of the receptor gene products as a function of axial position, and these cannot be obtained for the individual EphA proteins. A typical *in situ* hybridization signal for *EphA6* mRNA is illustrated in Fig. 1b, and *EphA6* mRNA quantification across the NT (x) axis is illustrated in Fig. 1c.

We similarly measured the *EphA4* and *EphA5* mRNAs (Fig. 1d). Like the *EphA6* gradient, that for *EphA5* is nonlinear. When the

EphA5 and *EphA6* points at each axial position are summed, the composite C_v curve that is generated is well fitted by equation (2) (see Box 1), in which C_{v0} , the composite value of *EphA5* + *EphA6* at the nasal pole of the retina ($x = 0$), is 0.26, and $k = 0.023$. (Curve fits were performed with Kaleidagraph, from Synergy Software.) In keeping with previous qualitative assessments^{4,10,18,19}, we found that RGC expression of *EphA4* mRNA is approximately constant across the NT axis (Fig. 1d). Adding the values at each point of the individual *EphA4*, *EphA5* and *EphA6* curves yields ΣEphA , the aggregate EphA receptor mRNA level per RGC (Fig. 1d, e). The ΣEphA points are well fitted by equation (4) (see Box 1), in which 0.26 is C_{v0} , 0.023 is k , and 1.05 is C_4 of equation (3) (Fig. 1e). The computer-selected C_4 constant is in close agreement with the mean measured value of *EphA4* mRNA (1.08 ± 0.07 ; Fig. 1d), and the selected C_{v0} constant is in similarly close agreement with the measured nasalmost *EphA5*+*EphA6* mRNA value of 0.30 (Fig. 1d). Thus, the ΣEphA receptor gradient of the mouse retina is indeed modelled by an exponential plus a constant. (See Supplementary Fig. 1 for additional data and discussion.) Note that this gradient is shallow: the difference in ΣEphA between the temporal and nasal poles of the retina is only about 2.75-fold (Fig. 1e).

We also quantified *EphA3* expression in the *EphA3*⁺ RGCs of P1 *Isl2-EphA3* heterozygous and homozygous knock-ins. We found that the *EphA3* mRNA expressed through the *Isl2* knock-in is approximately constant, and that the heterozygote signal is about 50% of the homozygote signal (Fig. 2a). When the measured *EphA3* ‘spikes’, corresponding to the subset of RGCs that are *Isl2*⁺ (ref. 4), are added to the wild-type ΣEphA curve, the quasi-oscillatory EphA gradients of the *Isl2-EphA3* heterozygotes (Fig. 2b) and homozygotes (Fig. 2c) are obtained.

The retinocollicular maps of these mice have been determined previously⁴, by using focal retinal injections of the fluorescent axonal tracer 1,1'-diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Fig. 2d). These label a small cluster of RGCs, which in the *Isl2-EphA3* mice will contain both *EphA3*⁺ and *EphA3*[−] neurons, together with their projecting axons and

Box 1

Relative signalling equations

+ / +	$\frac{dC_v}{dx} = kC_v$	(1)
+ / +	$C_v(x) = C_{v0}e^{kx}$	(2)
+ / +	$C(x) = C_{v0}e^{kx} + C_4$	(3)
+ / +	$C(x) = 0.26e^{0.023x} + 1.05$	(4)
ki / +	$C(x) = 0.26e^{0.023x} + 1.98$	(5)
ki / ki	$C(x) = 0.26e^{0.023x} + 2.91$	(6)
ki / +	$R_{lrs}(x) = \frac{0.26e^{0.023x} + 1.98}{0.26e^{0.023x} + 1.05}$	(7)
ki / ki	$R_{lrs}(x) = \frac{0.26e^{0.023x} + 2.91}{0.26e^{0.023x} + 1.05}$	(8)
ki / + A4 ^{+/−}	$C(x) = 0.26e^{0.023x} + 1.44 \text{ A3}^+$	(9)
ki / + A4 ^{+/−}	$C(x) = 0.26e^{0.023x} + 0.51 \text{ A3}^-$	(10)
ki / + A4 ^{+/−}	$R_{lrs}(x) = \frac{0.26e^{0.023x} + 1.44}{0.26e^{0.023x} + 0.51}$	(11)
+ / +	$R_{grs}(x) = \frac{3.7}{0.26e^{0.023x} + 1.05}$	(12)

C = concentration of ΣEphA (EphA4 + 5 + 6) per RGC; k = constant of proportionality; C_v = concentration of EphA5 + 6 per RGC; $C_{v0} = C_v$ at $x = 0$ (nasal pole of retina) (temporal pole $x = 100$); C_4 = concentration of EphA4 per RGC (constant); R_{lrs} = local relative signalling ratio; R_{grs} = general relative signalling ratio; ki = knock-in.

termination zones (TZs) in the SC⁴ (Fig. 2d). The map of *Isl2-EphA3* heterozygotes is duplicated for the nasalmost 76% of the NT axis, but collapses to a single coherent map for the remaining 24% of the retina⁴. That is, adjacent *EphA3*⁺ and *EphA3*[−] RGCs project to separate caudal–rostral collicular positions, with the TZs of *EphA3*[−] cells located more caudally, for all injections performed over the first 76% of the NT axis (Fig. 2d). In more temporal locations (77–100% of the axis), adjacent *EphA3*⁺ and *EphA3*[−] RGCs project to a single TZ⁴. Mapping ‘collapse’ is proposed to occur at 76% of the retinal axis because at this point the Σ EphA expression ratio between adjacent *EphA3*⁺ and *EphA3*[−] RGCs becomes too small for the mapping system to discriminate⁴; in other words, this ratio, which we refer to as the local relative signalling ratio (R_{LRS}), falls below a discrimination limit at this point. (Note that the absolute difference between adjacent *EphA3*⁺ and *EphA3*[−] RGCs does not vary across the NT axis of the knock-ins (Fig. 2a–c).) The quantification of receptor mRNAs allowed us to determine the value of this discrimination limit. The single on/off Σ EphA gradient in the heterozygous knock-ins (Fig. 2b, inset) can be modelled by two smooth-component exponentials—one for the

wild-type *EphA3*[−] RGCs (equation (4)), and a second for the knock-in *EphA3*⁺ RGCs. The equation for the *EphA3*⁺ curve is equation (5) (see Box 1), which has the constant heterozygote level of *EphA3* mRNA (0.93 ± 0.06) added to the C_4 constant of equation (4) (Fig. 2b). Similarly, the exponential that models the *EphA3*⁺ RGCs in the homozygous knock-ins is equation (6) (see Box 1), where the last constant reflects a further dose of *EphA3* (Fig. 2c).

Dividing equation (5) by equation (4) yields equation (7), the local relative signalling (RS) ratio function for the heterozygous knock-ins. This function, plotted in Fig. 2e, describes how the Σ EphA expression ratio for adjacent (formally, superimposed) *EphA3*⁺ and *EphA3*[−] RGCs varies with position along the NT axis. A collapse point at 76% of the NT axis defines a discrimination limit ratio of 1.36 on this curve (Fig. 2e). If this value is fixed for mice of any genotype, it should not be reached in the *Isl2-EphA3* homozygotes, because these mice display a duplicated retinocollicular map across their entire NT axis⁴. The function that describes R_{LRS} between adjacent *EphA3*⁺ and *EphA3*[−] RGCs in the homozygous knock-ins is equation (8), which is equation (6) divided by

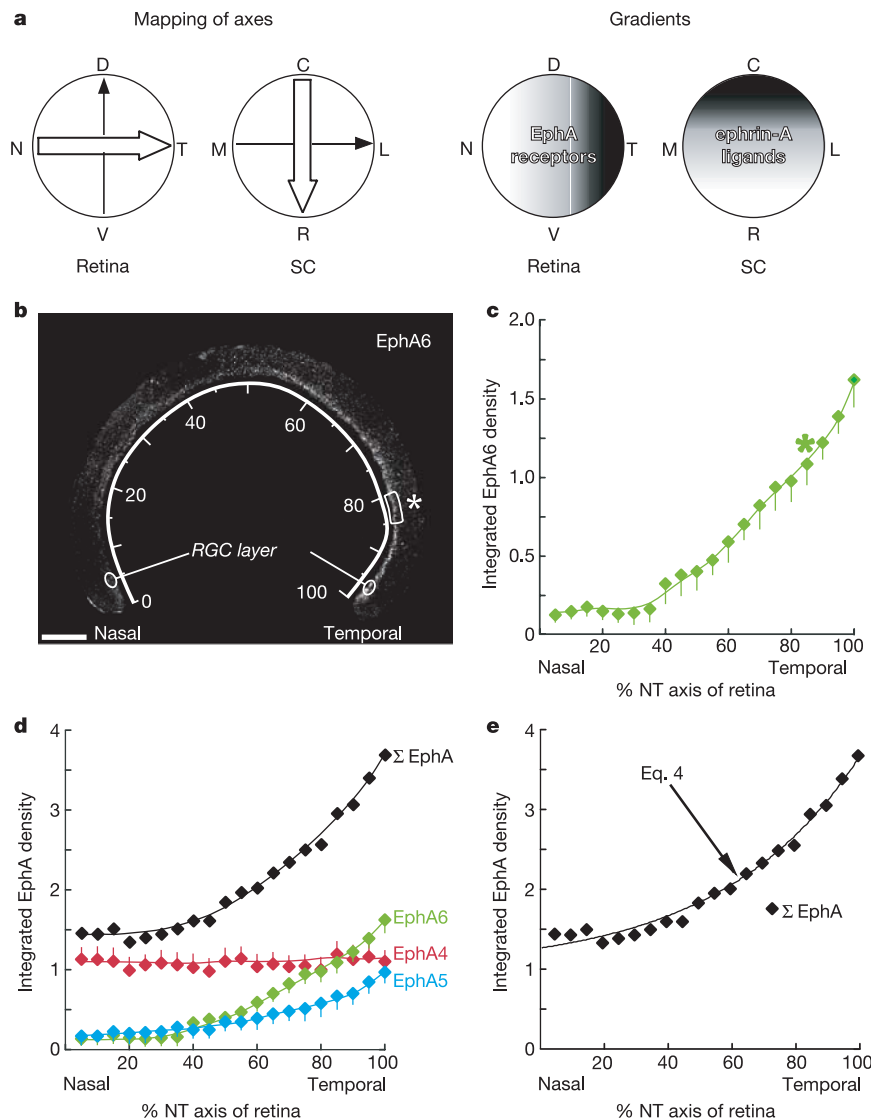


Figure 1 *EphA* mRNAs in the retina. **a**, Mapping of the dorsal–ventral (DV) and nasal–temporal (NT) retinal axes onto the lateral–medial (LM) and caudal–rostral (CR) axes of the SC (left); and *EphA* receptor and ephrin-A ligand gradients (right). **b**, Section of P1 mouse retina hybridized with an *EphA6* probe. Quantification was performed for 20 segments of the RGC layer (see Methods), one of which is outlined (asterisk). Scale bar, 200 μ m.

c, *EphA6* gradient (integrated signal density for *EphA6*⁺ RGCs per segment) averaged over seven sections comparable to that in **b**. Asterisk corresponds to the segment in **b**. **d**, *EphA4* ($n = 8$), *EphA5* ($n = 7$) and *EphA6* ($n = 7$) mRNAs, and their summed values (Σ *EphA*). Error bars in **c** and **d** show one standard deviation from the mean. **e**, The Σ *EphA* gradient is well fitted by equation (4).

equation (4) (see Box 1). The plot of equation (8) does indeed fail to reach the discrimination limit (Fig. 2f), and thus predicts the fully duplicated map.

We next used mouse knockouts of the *EphA4* gene²⁰ to generate compound mutants that are trans-heterozygous for both *Isl2-EphA3* and loss of *EphA4*. Removing half of the *EphA4* component from both the numerator and denominator of the local RS ratio in the heterozygous knock-ins predictably increases the value of this ratio across the NT axis (Fig. 3). The Σ EphA gradient of *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice is illustrated in Fig. 3a. The continuous functions that model the *EphA3*⁺ and *EphA3*⁻ components of this

gradient are equations (9) and (10) (see Box 1). Dividing equation (9) by equation (10) yields equation (11), the local RS ratio function for these mice. Given a discrimination limit ratio of 1.36, this function predicts that the *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} map should be largely duplicated but should still collapse, that the collapse point should be shifted temporally relative to that of *Isl2-EphA3* heterozygotes that are wild type for *EphA4*, and that it should occur at about 88% of the retinal NT axis (Fig. 3b). We generated these *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice and measured their retinocollicular map (Fig. 3c–e). This map is indeed duplicated throughout most of the retinal axis, but still collapses (Fig. 3e). The collapse point is shifted

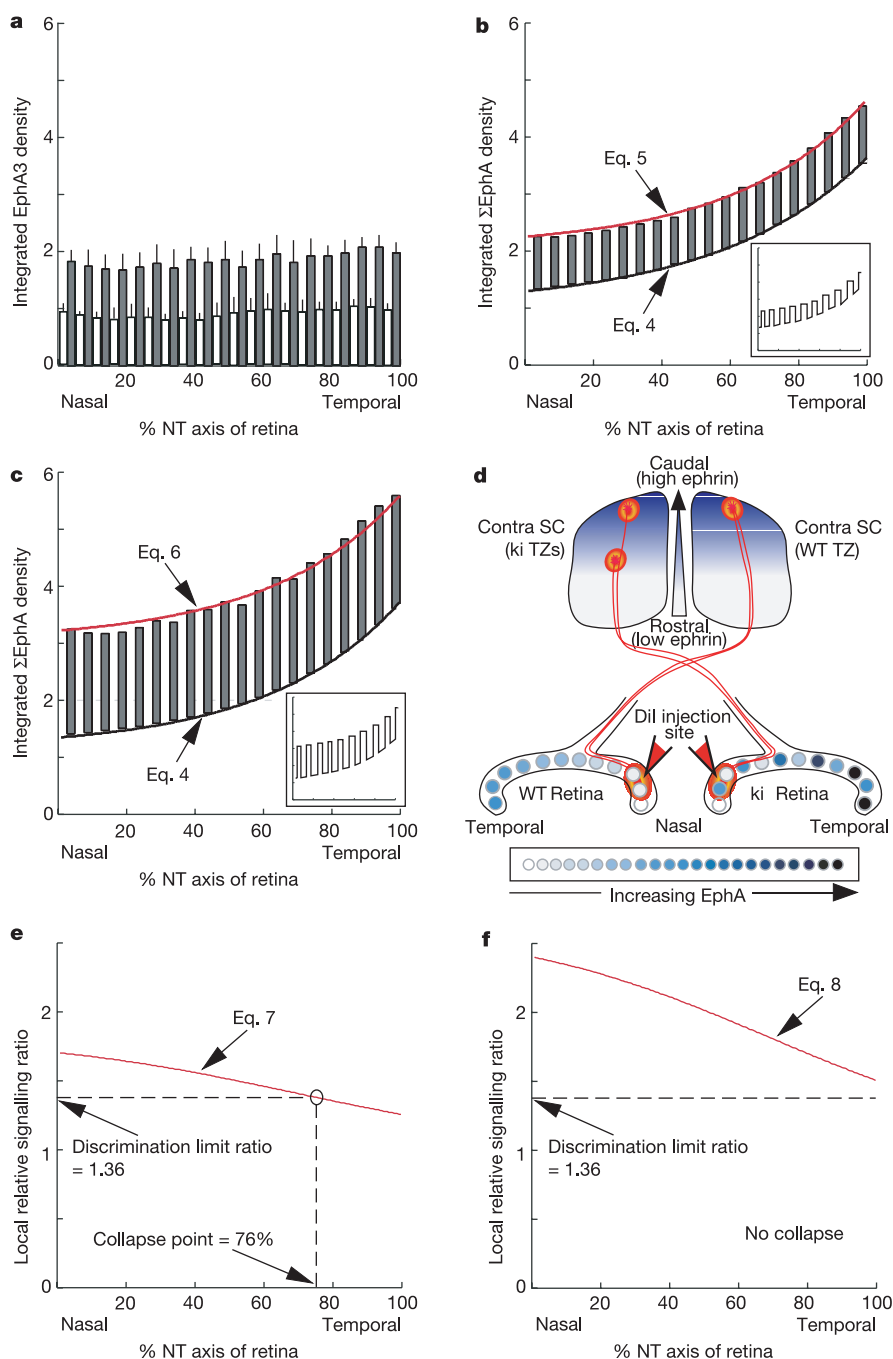


Figure 2 *EphA* expression in the knock-ins. **a**, Discontinuous RGC expression of *EphA3* mRNA in homozygous (*ki/ki*, grey bars) and heterozygous (*ki/+*, white bars) knock-ins. Error bars show one standard deviation from the mean ($n = 7$ and $n = 4$ measurements per point for *ki/ki* and *ki/+*, respectively). **b**, Σ EphA gradient in *Isl2-EphA3* heterozygotes (*ki/+*). The *ki/+* *EphA3* 'spikes' in **a** are superimposed on the curve of equation (4). The red curve (equation (5)) connects the tops of the spikes. The Σ EphA gradient shape is

approximated by the inset. **c**, Σ EphA gradient in *Isl2-EphA3* homozygotes (*ki/ki*). **d**, Results of Dil injection into the nasal retina of wild-type (WT, left) versus knock-in (*ki*, right) mice. Adjacent WT RGCs with equivalent EphA project to one TZ in the caudal SC, whereas adjacent *ki* RGCs with different levels of EphA project to two TZs, the more rostral of which corresponds to *EphA3*⁺ RGCs⁴. **e**, **f**, Local RS ratio function in *Isl2-EphA3* heterozygotes (*ki/+*, **e**) and in homozygotes (*ki/ki*, **f**).

temporally relative to that observed in heterozygous knock-ins that are wild type for *EphA4*, and now occurs at about 88% of the retinal axis (Fig. 3e).

Note that whereas the separation between *EphA3*⁺ and *EphA3*[−] TZs in the nasal retinae of *Isl2-EphA3*^{ki/+}*EphA4*^{+/+} mice is only on the order of 20% of the caudal–rostral axis of the SC, TZ separation in the *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice is much larger (Fig. 3e). This results in the lower map, which corresponds to the TZs of *EphA3*⁺ RGCs, being compressed into the rostral SC (see Supplementary Fig. 2 and accompanying discussion). This phenomenon is also predicted by relative signalling. At the nasal pole of the retinae of these mice, the Σ EphA ratio between adjacent *EphA3*⁺ and *EphA3*[−] RGCs is 2.2 (Fig. 3b), which is 69% of the operating range of the wild-type retina (1.0–2.75; Fig. 1d). In close correspondence, the separation of the nasalmost pair of duplicated TZs in Fig. 3e is 75% of the collicular axis. The large increase in TZ separation seen in the *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice also has important implications for proposed roles for ephrin-A ligands that are expressed in the retina²¹ (see Supplementary Information for discussion).

The R_{Irs} functions for each of the three remaining compound genotypes (Fig. 4a, c) make multiple additional predictions, all of which are fulfilled. The R_{Irs} function for *Isl2-EphA3*^{ki/+}*EphA4*^{+/-}

mice, for example, predicts a retinocollicular map that is duplicated across nearly the entirety of the retinal NT axis but collapses at the temporal pole (Fig. 4a). This prediction is fulfilled (Fig. 4d). Similarly, the maximal operating range ratio for wild-type mice is exceeded in the nasalmost 30% of the retina in these mice (Fig. 4a), which predicts TZ separations that approach the full extent of the caudal–rostral collicular axis. This is also true (Fig. 4d). The R_{Irs} functions of all three genotypes that are homozygous for *Isl2-EphA3* fail to reach the discrimination limit ratio, irrespective of their *EphA4* genotype (Figs 2e and 4b, c, respectively), and all are therefore predicted to exhibit fully duplicated, non-collapsing maps. Each of these predictions is also fulfilled (Fig. 4e, f; and Fig. 5c in ref. 4).

In the knock-ins, the local RS ratio, which defines a fold difference in Σ EphA between adjacent *EphA3*⁺ and *EphA3*[−] RGCs, accurately predicts the incidence and nasal–temporal position of mapping collapse, as well as the extent of map duplication. However, the local RS ratio in wild-type mice is only trivially different from 1.0, because adjacent RGCs along the wild-type axis have essentially the same level of Σ EphA. So how is it possible for local RS comparisons to be made in the context of the ensemble-wide competition that leads to formation of the wild-type retino-

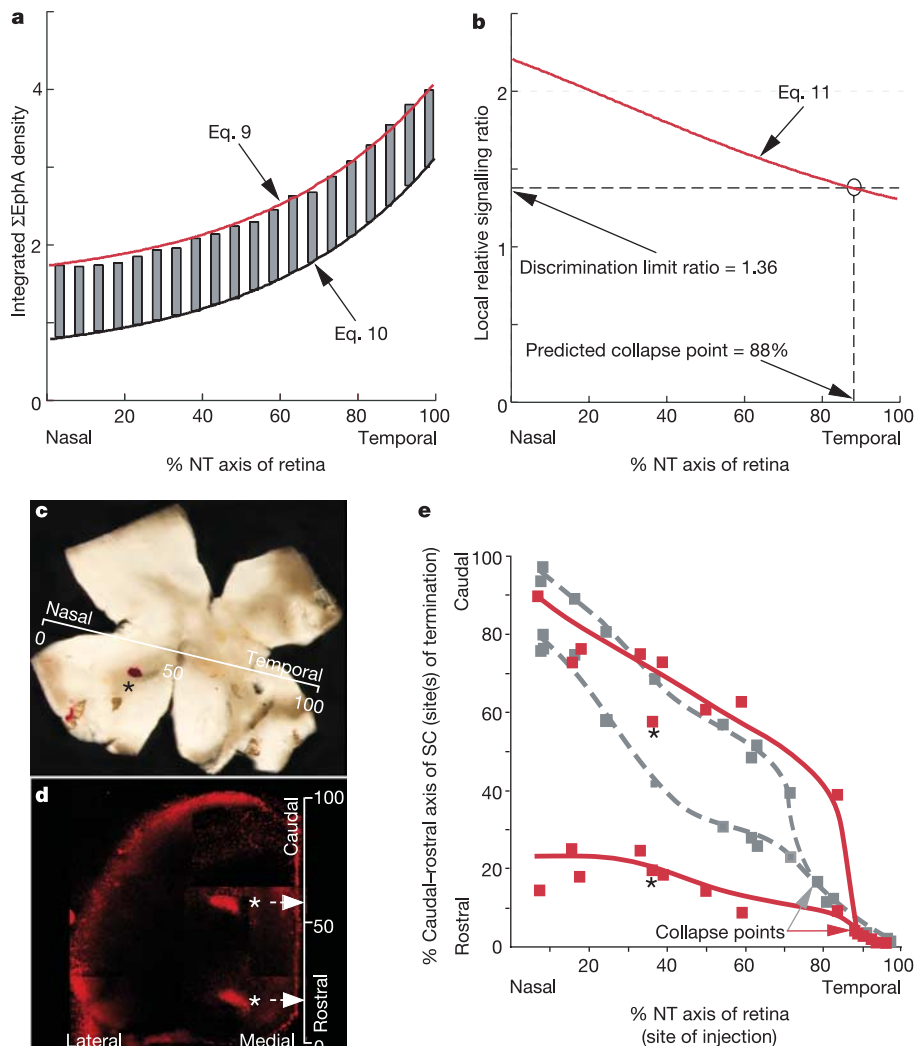


Figure 3 Map perturbation in *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice. **a**, Σ EphA gradient in *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice. **b**, Σ EphA ratio between adjacent *EphA3*⁺ and *EphA3*[−] RGCs, as a function of retinal position, in these compound heterozygotes. **c**, A P8 retina (flatmount) from these mice, with Dil injected into the ventral nasal quadrant, at 37% of the NT axis (asterisk). **d**, Two TZs (asterisks), at 20% and 58% of the caudal–rostral axis

(arrows), labelled in the SC contralateral to the retina in **c**. **e**, Retinocollicular map of *Isl2-EphA3*^{ki/+}*EphA4*^{+/-} mice (red points and curves), determined as in **c** and **d**. Asterisk points correspond to the TZs in **d**. Grey points and curves are from *Isl2-EphA3*^{ki/+}*EphA4*^{+/+} mice⁴. Scale bar, 1 mm.

collicular map? Map formation in most vertebrates takes place during a week-long period of competition between RGC axons for innervation targets in the SC^{17,22,23}. If ratiometric comparisons of ΣEphA are used to set the rules by which RGCs compete for a limiting feature of the SC during this time, the dominant cell in the ensemble will be the neuron with the highest levels of ΣEphA signalling and the greatest sensitivity to the collicular ephrin-As; that is, the RGC located at the temporal pole of the retina. (Temporal RGCs are indeed most sensitive to chemorepulsion by ephrin-As^{13,19,22,24}.) In this milieu, all RGCs might compare themselves with all other RGCs by calculating a general signalling ratio relative to this same temporal reference. (A compelling case for a dominant polar reference cell in 'competition by exclusion' models of retinotectal mapping was—on purely theoretical grounds—made nearly 30 years ago²⁵.) The highest temporal/nasal ΣEphA ratio (3.7:1.35, or 2.75; Fig. 1e) will characterize RGCs at the nasal pole. Increasingly more temporal RGCs will have increasingly lower ratios, reaching 1.0 at the temporal pole. Most importantly, our model requires that this general relative signalling ratio function provide the algorithm for retinocollicular mapping in wild-type mice. It is expressed by equation (12) (see Box 1), in which the ΣEphA value at the temporal pole of the mouse retina (3.7; Fig. 1e) is divided by the term on the right-hand side of equation (4), which specifies the ΣEphA gradient across the NT axis.

The curve described by equation (12) is plotted in Fig. 5. Although in reality sigmoidal, it is remarkably close to the linear configuration of the wild-type mouse map. When the data points that were used previously to plot this map⁴ are superimposed on the curve, there is a nearly perfect correspondence (Fig. 5). Thus, the

wild-type retinocollicular map in the mouse is accurately derived from this simple relative signalling rule. It is particularly important to note that the local RS ratio functions of equations (7), (8) and (11) are merely specialized applications of the general RS ratio function of equation (12): the same discrimination limit (1.36) holds for wild-type mice, and the same general ratioing operation applies to the local determination of mapping collapse in the knock-ins, because for two cells RGC_1 and RGC_2 , $(3.7/\Sigma\text{EphA}_1)/(3.7/\Sigma\text{EphA}_2) = \Sigma\text{EphA}_2/\Sigma\text{EphA}_1$.

Our analyses strongly support the hypothesis that retinocollicular mapping works through relative signalling. By employing the same temporal reference, all RGCs effectively perform a ΣEphA ratio comparison between themselves and all other RGCs. Translation of this ratiometric comparison into competition between RGC axons in the SC might be achieved by one of several mechanisms. One possibility, involving ΣEphA regulation of RGC competition for brain-derived neurotrophic factor, is described in Supplementary Fig. 3. Note that the reciprocal mapping algorithm of equation (12) accounts for the ability of the wild-type map to discriminate between adjacent RGCs whose local relative signalling ratios are near 1.0: all local comparisons are made in the context of a global comparison with the temporal reference. As we will describe elsewhere, this algorithm also yields the retinocollicular maps of the knock-ins. Although the general RS algorithm has considerable predictive power, our data suggest that ΣEphA gradients on their own do not produce a map that is perfect: in moving nasally from the temporal pole of the retina, the RS discrimination limit of 1.36 is not reached until about 85% of the retinal axis (Fig. 1d). Previously described refinement mechanisms such as coordinated electrical

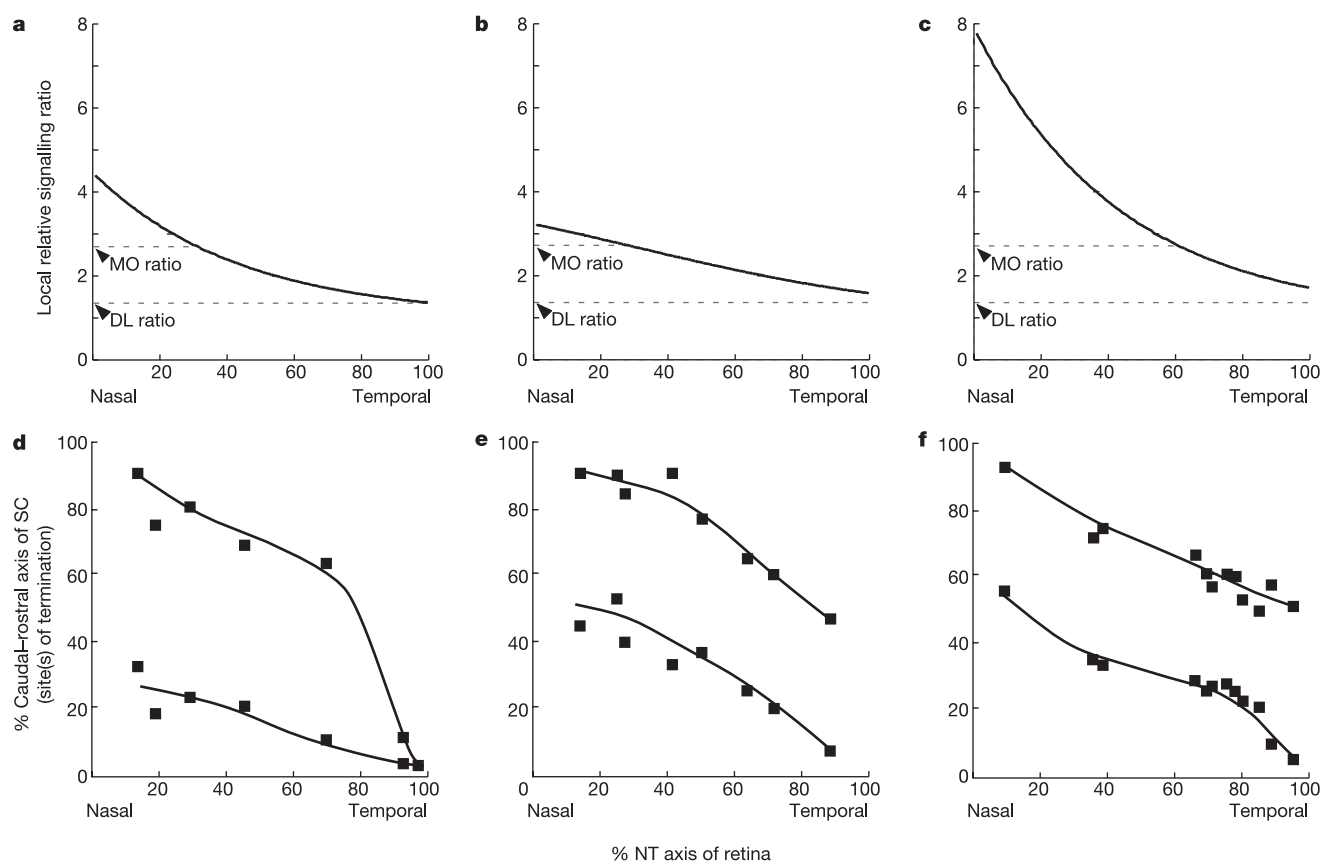


Figure 4 R_{rs} functions and measured retinocollicular maps in all possible $Isl2\text{-}EphA3/EphA4$ compound genotypes. **a–c**, R_{rs} functions for $Isl2\text{-}EphA3^{ki/+}EphA4^{-/-}$ (**a**), $Isl2\text{-}EphA3^{ki/ki}EphA4^{+/-}$ (**b**) and $Isl2\text{-}EphA3^{ki/ki}EphA4^{-/-}$ (**c**) mice were calculated by using the measurements and formulations described in the text. For **a**, $R_{rs}(x) = (0.26e^{0.023x} + 0.90)/0.26e^{0.023x}$; for **b**, $R_{rs}(x) = (0.26e^{0.023x} + 2.31)/(0.26e^{0.023x} + 0.54)$; for **c**, $R_{rs}(x) = (0.26e^{0.023x} + 1.80)/0.26e^{0.023x}$. **d–f**, Maps for these three genotypes (**d**, **e** and **f**, respectively) were measured by using the methods illustrated in Fig. 3. Discrimination limit (DL; 1.36) and maximal operating (MO; 2.75) ratios are indicated in **a–c**.

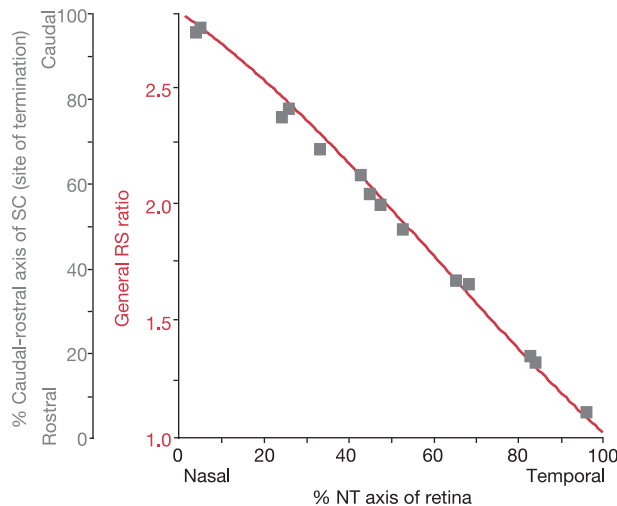


Figure 5 RS derivation of the wild-type map. The red curve (equation (12)) displays the ratio of the Σ EphA value at the temporal pole of the mouse retina (3.7; Fig. 1d) relative to the Σ EphA value across the NT axis. This general RS ratio varies from a nasal maximum of 2.75 to a temporal minimum of 1.0. The offset y axis (grey) is the rostral–caudal axis of the SC, and is used to display the previously determined data points of the wild-type retinocollicular map⁴.

activity²⁶, which are unaltered in our mice, must therefore operate to provide ultimate topographic precision.

Gradients of EphA receptors and their ligands are thought to mediate the topographic wiring of multiple other sensory maps in the developing central nervous system^{27,28}. More generally, many aspects of neural development, including the survival of neurons based on access to neurotrophins and the stabilization of synapses in the neocortex²⁹, depend on competition between cells within a developing ensemble. Like retinocollicular innervation, these phenomena are typically dynamic and take place over an extended period. They might also be governed by relative signalling. □

Methods

Quantitative expression analysis

In situ hybridizations with ³³P-radiolabelled cRNA probes were performed as described previously³⁰. EphA3, EphA4, EphA5 and EphA6 probes were made to the equivalent amino-terminal regions of all four receptors. All probes were between 862 and 927 base pairs in length, contained between 25% and 27% UTP, and were radiolabelled to the same specific radioactivity. Hybridizations were performed at 10⁵, 1.5 × 10⁵ and 2 × 10⁵ c.p.m. per slide; these yielded linear increases in signal density across the full NT axis of the retina over the exposure times we employed. Equal probe concentrations were hybridized to adjacent 16-μm horizontal retinal sections, and then emulsion-dipped, developed, and photographed under identical conditions.

Digital images of *in situ* hybridizations were taken at 1,300 × 1,300 resolution. For measurement of signals, the NT axis of the RGC layer of the retina was divided into 20 equal segments; these segments were quantified with the Scion Software (NIH Image version for PC, <http://www.scioncorp.com>). Positive *in situ* hybridization greyscale signal was counted after removal of a background signal, which was preset after measurement of control sense probe signals by using 'density slicing'. This lower grey detection signal was set at 50 for all quantifications. We performed a more limited set of control measurements with a lower grey threshold of 30, which showed a linear optical response over a 2.5-fold range of detected signal. Using this 30 lower grey threshold, the temporal/nasal fold difference in Σ EphA expression in the retina was, despite the higher absolute signal values, again 2.75-fold. Pixel counts are given in units of 'integrated signal density' (isd, referred to as 'integrated density' on page 37 of the online Scion Image manual). The isd corresponds to the sum of greyscale values for all pixels above the lower grey detection threshold in a given segment (background subtracted). Within a segment, only pixels with signal values above this threshold were scored. Thus, the average area of signal quantification in each segment for hybridizations with EphA4, EphA5 and EphA6 probes—which detect mRNAs expressed by all RGCs—was approximately twice that of the average area for EphA3, which is expressed in about 50% of RGCs in the knock-ins. Neither control sense probes nor regions of the retinal sections that were devoid of cells yielded a positive score using the more than 30 lower grey threshold. The isd values of all plots in this paper are based on the same signal threshold, scale and quantification parameters, and can be compared directly.

Anterograde axon labelling

Focal injections of Dil (Molecular Probes) were made into the left retinae of P7–P8 mice; TZs in the contralateral SC labelled by these injections were analysed 24 h later, as described previously^{4,23}. Injected retinae and contralateral SCs were scored, blind to genotype, as whole mounts.

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A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*

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All animals exhibit innate behaviours in response to specific sensory stimuli that are likely to result from the activation of developmentally programmed neural circuits. Here we observe that *Drosophila* exhibit robust avoidance to odours released by stressed flies. Gas chromatography and mass spectrometry identifies one component of this '*Drosophila* stress odorant (dSO)' as CO₂. CO₂ elicits avoidance behaviour, at levels as low as 0.1%. We used two-photon imaging with the Ca²⁺-sensitive fluorescent protein G-CaMP to map the primary sensory neurons governing avoidance to CO₂. CO₂ activates only a single glomerulus in the antennal lobe, the V glomerulus; moreover, this glomerulus is not activated by any of 26 other odorants tested. Inhibition of synaptic transmission in sensory neurons that innervate the V glomerulus, using a temperature-sensitive *Shibire* gene (*Shi^{ts}*), blocks the avoidance response to CO₂. Inhibition of synaptic release in the vast majority of other olfactory receptor neurons has no effect on this behaviour. These data demonstrate that the activation of a single population of sensory neurons innervating one glomerulus is responsible for an innate avoidance behaviour in *Drosophila*.

We observed that *Drosophila* tend to avoid chambers in which other flies have previously been subjected to stress by mechanical shaking or electric shock. To investigate the basis of this behaviour, about 70 'emitter' flies were subjected to mechanical stress by vortexing them in a test tube (see Methods). We removed the stressed flies, and allowed naive 'responder' flies to choose between this 'conditioned tube' and a fresh tube, in a T-maze apparatus². The majority (80–95%) of responder flies avoided the conditioned tube in a one-minute trial (Fig. 1a). A similar avoidance response was observed when emitter flies were stressed using electric shock (Fig. 1a). To determine whether the mere presence of flies in a tube causes emission of the avoidance-promoting substance, flies were gently introduced into a tube using positive phototaxis, and removed after several hours of occupancy. Despite the evident presence in the tube of fly waste, responder flies showed no avoidance response to the tube (Fig. 1a, 'no stress'). This suggests that avoidance is elicited by a substance emitted in response to mechanical or electrical stress. The emission of the substance is not observed when anaesthetized flies are vortexed, indicating that such emission requires neural activity.

Surgical removal of the third antennal segment, which houses the olfactory receptor neurons (ORNs), eliminated the avoidance response (Fig. 1b). By contrast, removal of the arista or maxillary palps had no effect. These data suggest that the olfactory system mediates the avoidance response. We therefore operationally refer to the substance evoking the avoidance response as *Drosophila* stress odorant (dSO).

Olfactory sensory neurons in the antennae project axons to glomeruli in the antennal lobe. Projection neurons then connect the antennal lobe to the mushroom body and lateral protocerebrum³. Conditioned olfactory avoidance responses produced

by associative learning require the mushroom body⁴, whereas unconditioned avoidance responses to chemical repellents do not^{5,6}. We asked whether the mushroom body is required for the avoidance response to dSO, by ablating this structure through hydroxyurea (HU) treatment at a critical stage of development⁷. Alternatively, we have used a mushroom-body-specific Gal4 enhancer trap line to drive the expression of UAS-*Shi^{ts}*, a dominant-negative mutant of dynamin that inhibits neurotransmitter release at a non-permissive temperature¹. Neither treatment impaired the avoidance response to dSO (Fig. 1c), although a deficit in olfactory learning confirmed that the lesion was successful (Fig. 1c, lower panel). These data suggest that the avoidance response to dSO does not require brain structures necessary for learned olfactory avoidance.

Analysis of the chemical components of dSO by gas chromatography and mass spectrometry (GC/MS) revealed a small peak of 44 daltons that corresponds to CO₂, which was present in samples of air from conditioned tubes (Fig. 2a, left panel, arrow). Further analysis using a CO₂ respirometer indicated that flies emit about three- to fourfold more CO₂ during shaking than do undisturbed flies (Fig. 2b). By comparison to signals obtained by injecting known amounts of pure CO₂ into the respirometer, the concentration of CO₂ in conditioned tubes was estimated at ~0.2% (data not shown). We next asked whether CO₂ alone evoked avoidance behaviour in a T-maze assay. Flies avoid CO₂ in a dose-dependent manner, at concentrations far below anaesthetic levels (30%) (Fig. 2c; see also Supplementary Fig. S1). A concentration as low as 0.1% above the ambient CO₂ level (0.0376%) evoked a statistically significant avoidance response (performance index, PI = 29.6 ± 10.9, *P* < 0.001 by ANOVA, *n* = 3, see Supplementary Fig. S1). A concentration comparable to that estimated in dSO (~0.2%), although it did elicit significant avoidance, evoked a weaker response than did dSO itself (Fig. 2c, '+ CO₂ 0.02 ml' versus 'CS shaken', where 'CS' indicates the wild-type Canton S *Drosophila* strain), suggesting that stressed flies release additional repellent compound(s) together with CO₂.

We analysed the pattern of glomerular activity in the antennal lobe elicited by CO₂, using a calcium-sensitive fluorescent indicator protein (GCaMP) and two-photon microscopy⁸. Electrophysiological recordings have previously identified CO₂-responsive ORNs in the ab1 basiconic sensilla of the antenna^{9–11}, but the receptor expressed in these neurons, and their projections, have not been established. Experiments in Lepidoptera^{12,13} and Diptera^{14,15} have traced the glomerular targets innervated by axons from sensilla containing CO₂-responsive ORNs, but these sensilla contain other ORNs as well. Moreover, the studies in Dipteran species have differed with respect to the number and identity of the glomeruli innervated^{14,15}. Ca²⁺ imaging permits an analysis of the glomerular activation pattern of CO₂-responsive sensory neurons in the antennal lobe. We first examined flies in which the GCaMP indicator (UAS-GCaMP) is driven in all neurons by the *Elav-GAL4* activator. At concentrations of CO₂ up to 10%, only the most ventral pair of glomeruli, the V glomeruli², were activated (Fig. 3a). Activation was detected by as little as 0.05% CO₂ (Supplementary Fig. S1). These glomeruli were not activated by any of 26 other odorants tested (data not shown).

We have previously shown¹⁶ that axonal projections to V originate from antennal sensory neurons expressing the candidate gustatory receptor GR21A (Fig. 3d, left). GR21A⁺ neurons are located in the dorso-medial portion of the antenna (Fig. 3d, right), the region where CO₂-responsive ab1 neurons are positioned in basiconic sensilla^{10,11}. Calcium imaging was thus performed with flies in which the UAS-GCaMP reporter was driven by a GR21A promoter-Gal4 activator (Fig. 3b). CO₂ (Fig. 3b), as well as air from a tube conditioned by traumatized flies (Fig. 3c, left), activated GR21A sensory termini in the V glomeruli. Air from a tube that had contained undisturbed flies produced significantly lower levels of

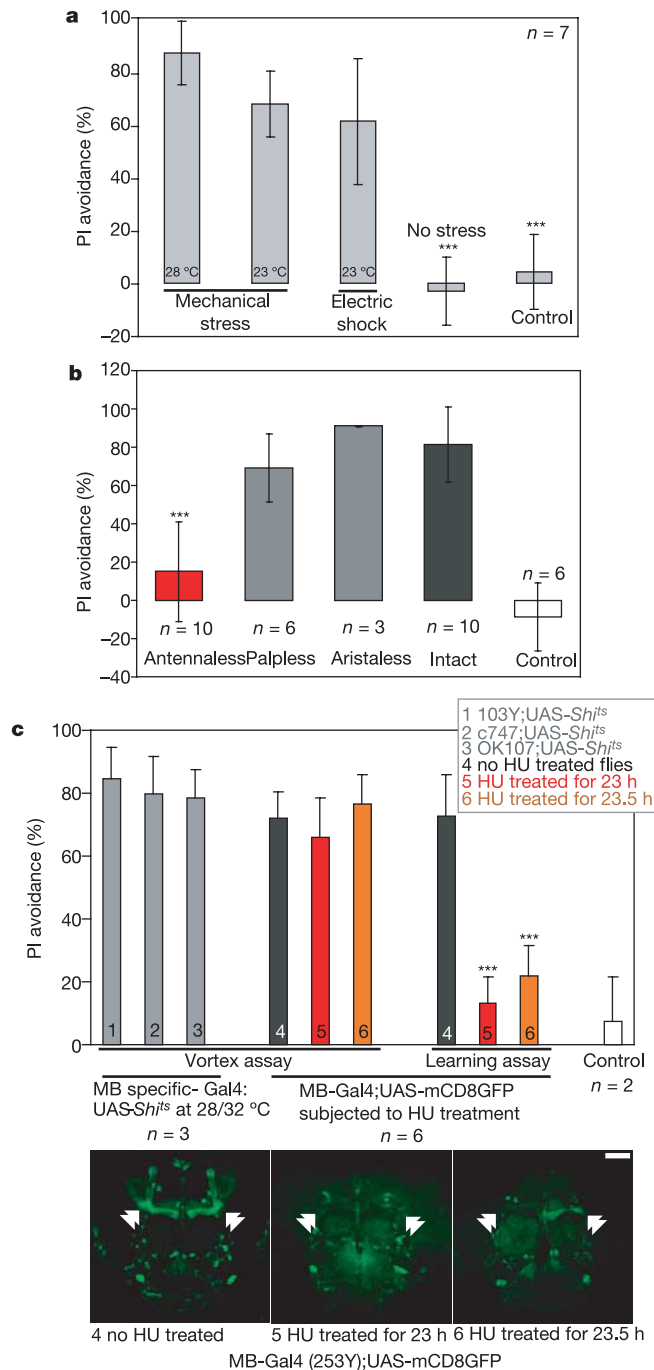


Figure 1 *Drosophila* exhibits innate avoidance of odorants released by stressed flies. **a**, Avoidance of tubes containing air from stressed flies in a T-maze, quantified as the PI (see Methods) of avoidance. The 'No stress' tube was conditioned by flies gently introduced and removed by phototaxis. The 'Control' is an empty tube. Bars indicate the mean $\pm$ s.e.m. of seven independent experiments. ***, $P < 0.001$ in this and all subsequent figures (ANOVA). **b**, Effect of ablating different sensory organs on dSO avoidance. **c**, dSO-avoidance is MB-independent. Three MB-specific Gal4 lines, 103Y (1; <http://www.fly-trap.org>), c747 (ref. 29) (2), and OK107 (ref. 29) (3), each crossed with UAS-*Shi*^{ts} and tested at 28 or 32 °C. Flies were also treated with HU (5, 6) or without HU (4) to chemically ablate the MB. The HU ablation prevents classical olfactory avoidance conditioning ('Learning assay'). Fluorescent micrographs indicate successful HU-mediated MB ablation (arrowheads), visualized in 253Y;UAS-mCD8GFP brains. Scale bar, 100 μm. Error bars in **b** and **c** indicate the s.e.m.

activation (Fig. 3c, right). These results indicate that both CO₂ and dSO activate neurons that express the GR21A receptor and project to the V glomerulus.

We next asked whether the GR21A⁺ sensory neurons are necessary for the avoidance responses to CO₂ and dSO. For this, we employed *Shibire*^{ts} to reversibly inactivate these neurons at increased temperature¹. Flies bearing either of two independent GR21A-Gal4 insertions and UAS-*Shi*^{ts} no longer exhibited the avoidance response to ~1% CO₂ at a non-permissive temperature (that is, a temperature at which neurotransmitter release cannot occur), but revealed a normal response at a permissive temperature (Fig. 3e, red versus blue bars labelled '2', respectively). Control flies expressing either of the GR21A-Gal4 drivers, but not UAS-*Shi*^{ts}, showed normal CO₂ avoidance at the non-permissive temperature (Fig. 3e). Furthermore, flies expressing UAS-*Shi*^{ts} and either of two Gal4 drivers broadly expressed in other ORNs, but not in GR21A⁺ neurons (OR83b-Gal4, expressed by ~80% of ORNs, or Or47b-Gal4; L. Vosshall, personal communication), exhibited normal CO₂ avoidance at the non-permissive temperature (Fig. 3e). Similarly, flies expressing UAS-*Shi*^{ts} and another Gal4 driver, GH146-Gal4, which is expressed in about two-thirds of antennal lobe projection neurons^{5,17} (but not in those innervating V), also showed robust CO₂ avoidance at the non-permissive temperature (Fig. 3e). These data indicate that GR21A⁺ sensory neurons that project to the V glomerulus are probably the sole population of ORNs responsive to

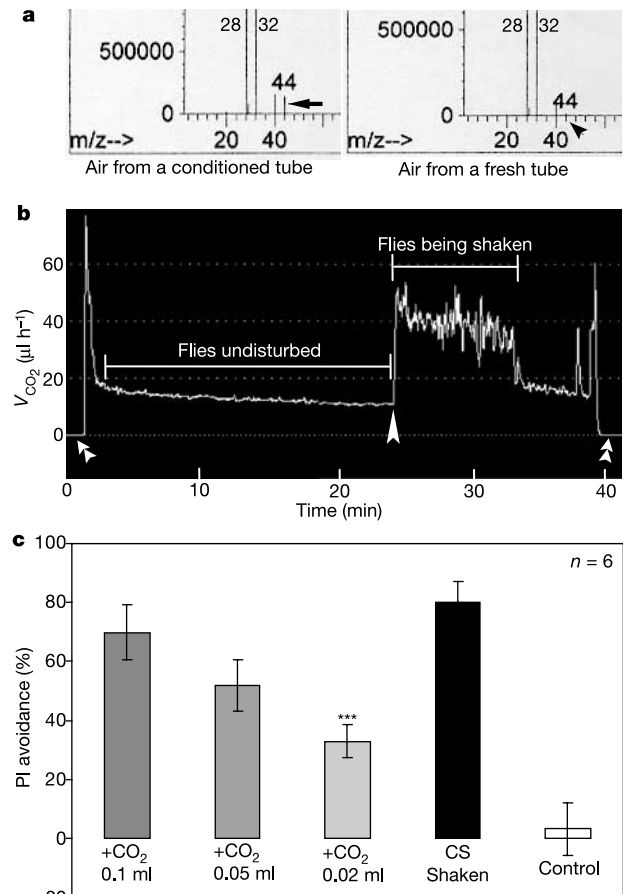


Figure 2 CO₂ is a component of dSO. **a**, Mass spectrometry of air sample derived from ~250 shaken flies. Arrow (44 daltons) indicates CO₂. **b**, Respirometer analysis of CO₂ emission from undisturbed and shaken flies. Double arrowheads indicate the CO₂ level of an unoccupied tube. **c**, Flies avoid CO₂ in a dosage-dependent manner. The indicated volumes of pure CO₂ were infused into a 15-ml tube immediately before the choice tests. 'CS shaken' indicates avoidance PI obtained with tube conditioned by ~70 Canton S flies. 'Control' indicates empty tube. Data represent mean $\pm$ s.e.m. ($n = 6$).

CO₂, and are required for the avoidance response. Flies expressing UAS-*Shi^{ts}* in GR21A⁺ neurons still avoided dSO (Fig. 3e, red bars labelled '1'). Although a reduced response was observed using one of the two driver lines (Fig. 3e, GR21aG4(2);UAS-*Shi^{ts}*), this reduction did not reach statistical significance. These data support the notion that dSO contains other repellent(s), in addition to CO₂.

To characterize further the neural substrates mediating dSO-responsiveness, we conducted a screen for UAS-*Shi^{ts}*-dependent

defects in dSO-avoidance, using a collection of Gal4 enhancer trap lines (K. Kaiser). A pilot screen of ~250 lines yielded 12 exhibiting reduced dSO avoidance at non-permissive temperatures (G.S.B.S., unpublished work). Several of these dSO-unresponsive lines also exhibited a strong and specific reduction in CO₂ avoidance in subsequent tests, including one designated c761 (Fig. 4a, b). Analysis of the c761 expression pattern revealed that it includes a subset of ORNs in the third antennal segment (Fig. 4c, right), but

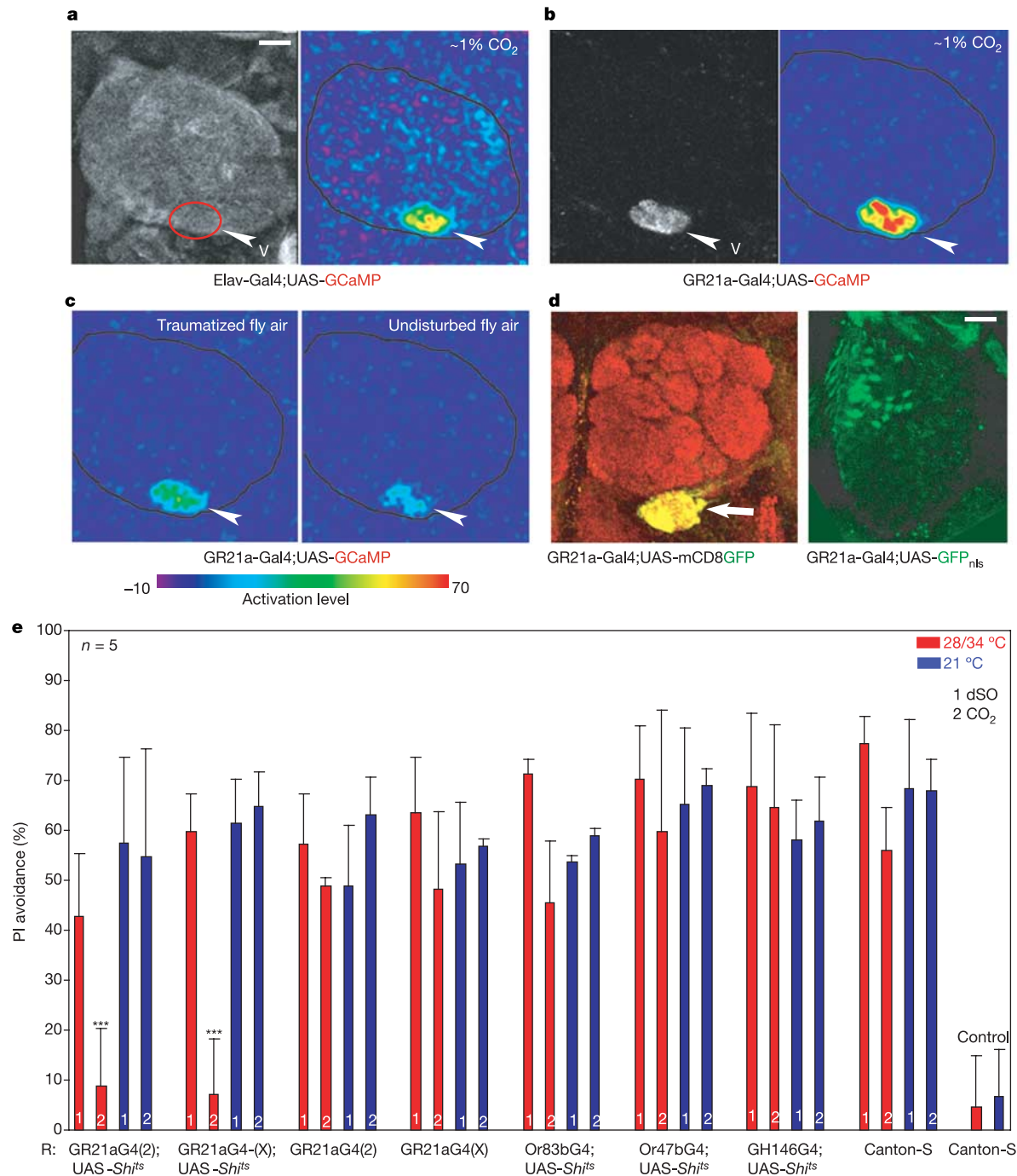


Figure 3 CO₂ avoidance is mediated by ORNs that project to the V glomerulus. **a**, Calcium response to CO₂ imaged using a pan-neuronally expressed GCaMP reporter. Left, prestimulation image; right, ~1% CO₂. Colour scale indicates activation level (red is the highest). Arrowheads in **a–c** indicate V glomerulus. Scale bar, 10 μm. **b**, Activation by CO₂ of presynaptic terminals of GR21A⁺ neurons innervating V glomerulus. **c**, Activation of GR21A⁺ neurons by dSO (left) versus control (right). **d**, GR21A⁺ ORNs project to V (arrow). Left, double-labelling of antennal lobe with anti-GFP and mAb nc82 (see also ref.

16). Right, GR21A⁺ sensory neuron cell bodies in the dorso-medial region of the antenna. Scale bar, 15 μm. **e**, Inhibition of synaptic transmission in GR21A⁺ neurons using *Shi^{ts}* blocks CO₂ avoidance. Red and blue bars indicate non-permissive and permissive temperatures, respectively. Bars labelled '1' and '2' are responses to dSO and CO₂, respectively, of flies of the indicated genotypes. ***, *P* < 0.001, by ANOVA. Error bars indicate the s.e.m.

not projection neurons (not shown). That this line is deficient in avoidance of CO₂, as well as of dSO, suggested that these ORNs might include those projecting to V, and others projecting to additional glomeruli. The projections of c761⁺ neurons to the antennal lobe were consistent with this expectation; labelling was observed both in the V glomerulus, and in several other glomeruli (Fig. 4c, left; Fig. 4d, left). Calcium imaging of c761;UAS-GCaMP flies revealed activation of V by CO₂ (Fig. 4d) as well as by dSO (not shown), confirming expression of this enhancer trap in GR21A⁺ neurons.

Together, these results indicate that c761 is expressed in, among others, CO₂-responsive sensory neurons that project to V (Fig. 4c). Because c761 was isolated in a screen for dSO-unresponsive lines, these data provide additional evidence that CO₂ is a behaviourally relevant component of dSO. Moreover, the observation that c761 expresses in additional populations of ORNs besides GR21A⁺ neurons suggests that these ORNs may respond to other active components of dSO.

We have shown that *Drosophila*, when stressed, emits an odorant mixture that elicits avoidance in other flies, and have identified CO₂ as one active component of this mixture. Calcium imaging data suggest that a single population of primary olfactory receptor neurons, which projects to the V glomerulus¹⁶, is activated by CO₂. Specific inhibition of neurotransmission in these GR21A⁺ sensory neurons, but not in other sensory neurons, abrogates CO₂ avoidance behaviour. Together, these data identify a single population of olfactory sensory neurons that mediates robust avoidance to a naturally occurring odorant, and provide initial insight into the neural circuitry that underlies this innate behaviour.

Many insect species, when stressed or threatened, emit semiochemicals that evoke aggressive or avoidance behaviour in conspecifics¹⁸. Whether *Drosophila* actually uses dSO to signal stress to

conspecifics in the wild, and the conditions under which they might do so, are not yet clear. We have used experimental stimuli such as mechanical agitation and electrical current to elicit release of dSO from *Drosophila*. Although such conditions are artificial, they afford us the ability to maintain tight control over the stimulus and the organism's response, as well as to apply molecular genetic tools to monitor and perturb neural activity. Although these stimuli increase physical and metabolic activity, it is possible that dSO could also be emitted in response to threats.

We have identified CO₂ as one active component of dSO. CO₂ is known to be an important chemical messenger for many insect species¹⁹. In mosquitoes, for example, CO₂ is an attractant that directs the insect towards warm-blooded animals²⁰. At all the concentrations of CO₂ that we tested, we detected only avoidance responses. The different behavioural responses to CO₂ exhibited by mosquitoes and *Drosophila* are likely to reflect hard-wired, species-specific differences in neural circuitry; nevertheless, we cannot exclude that these behavioural differences may be context-dependent. The behavioural and physiologic responses to CO₂ that we have measured in the laboratory are seen at several-fold increases above ambient (~0.0376%) that are well within the range measured for other insects¹⁹. The role of CO₂ in the ethology and ecology of *Drosophila* remains to be explored, but the highly specific olfactory circuitry revealed by our experiments suggests that it may be important.

Current data suggest that in *Drosophila*, most odorant compounds excite multiple populations of olfactory sensory neurons, each expressing a single olfactory receptor gene. A given odorant will therefore activate multiple glomeruli in the antennal lobe^{8,21}. By contrast, our data suggest that a single population of ORNs, and therefore a single glomerulus (V), are involved in sensing and avoiding CO₂. Previous electrophysiological studies identified ORNs uniquely responsive to CO₂, located exclusively in ab1

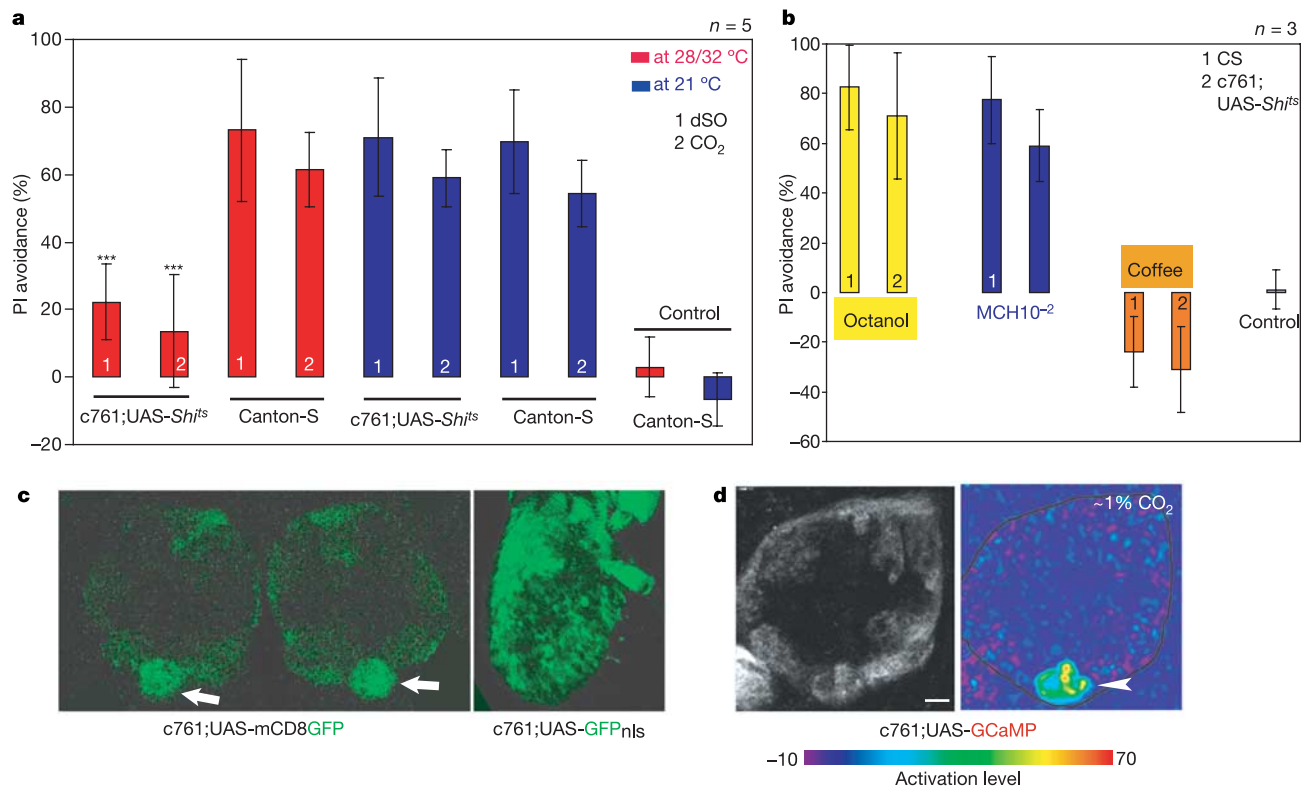


Figure 4 A dSO-unresponsive enhancer trap line is also defective in its CO₂ response. **a**, Response of c761;UAS-Shi^{ts} flies to dSO (labelled '1') or CO₂ (labelled '2') at the non-permissive (red bars) and permissive (blue bars) temperatures. ***, *P* < 0.001). **b**, Wild-type (1) and c761;UAS-Shi^{ts} (2) flies exhibit equivalent responses to other

repellents (yellow and blue bars) and an attractant (orange). **c**, c761-Gal4 is expressed in antennal sensory neurons (right panel, nuclear-GFP reporter) that project to V (arrows), and other glomeruli. **d**, Calcium imaging of CO₂ responses in c761;UAS-GCaMP flies reveals activation in V (arrowhead).

basiconic sensilla^{10,11}. These and the present data suggest that CO₂ activates a single population of ORNs, and that these ORNs respond only (or primarily) to CO₂. We cannot exclude, however, that other ORNs, (or antennal lobe projection neurons innervating glomeruli other than V), are also activated by CO₂ at levels below the detection limit of our imaging technology. Nevertheless, previous studies using this method have shown that multiple glomeruli are activated by most odorants tested⁸, so it is striking that just a single glomerulus is activated by CO₂. Furthermore, the behavioural response to CO₂ is extinguished by genetic silencing of GR21A⁺ sensory neurons. The fact that the avoidance response to CO₂ is unaffected by genetic silencing of Or83b or GH146 neurons further suggests that large populations of ORNs and projection neurons not directly innervating V are unnecessary for CO₂ avoidance. Taken together, these data suggest that a dedicated circuit, which involves a single population of ORNs, mediates detection of CO₂ in *Drosophila*. The simplicity of this early-stage olfactory processing offers a great advantage in further tracing the circuits that translate CO₂ detection into an avoidance response.

In general, recognition of many odorants in insects probably requires the decoding of combinatorial patterns of glomerular activation^{8,22–24}, perhaps combined with complex temporal dynamics in the antennal lobe²⁵. Nevertheless, our data suggest that there is also a set of olfactory stimuli, including CO₂, that release innate behaviours by activating a single class of primary sensory neurons and their associated glomerulus. In *Drosophila*, these stimuli may also include mating pheromones which, in other insect species, are known to activate specialized glomeruli^{26,27}. In *Caenorhabditis elegans*, the activation of a single chemosensory neuron can elicit a repulsive behaviour²⁸. Uni-glomerular circuits dedicated to the detection of certain odorants may have evolved to provide innate behavioural responses to these stimuli, which are essential to survival or reproduction of the species. □

Methods

Flies

Unless otherwise indicated, Canton-S flies (Caltech stock) were used for all experiments. Flies carrying the UAS-*Shi^{ts}* transgenes on the X and third chromosomes were reared at 20°C. For reversible neuronal silencing, flies were incubated in a 28°C room for ~90 min and then in a 32° or 34°C water bath for 5 min, before performing the behavioural experiments at 28°C. The permissive temperature used in experiments with *Shi^{ts}* was 21°C. To generate antennalless, palpless or aristaleless flies, extirpations were performed using fine tweezers under CO₂ anaesthesia, and the animals were allowed to recover for ~24 h before testing.

Behavioural tests

For behavioural testing, ~40 'responder' and 70 'emitter' adult flies (3- to 6-days post-eclosion, mixed gender) were anaesthetized using CO₂ and sorted into separate vials 24–48 h before use, or were sorted using an aspirator. A 16-inch 15-W fluorescent bulb was horizontally centred on the bench-top, behind the testing apparatus, in an otherwise dark room. Responder flies were transferred into the T-maze by first placing them into a 15-ml plastic tube (Fisher no. 149598), and tapping them into the elevator of the T-maze. While the responders were in the elevator, emitter flies were vortexed in a clean 15-ml tube in 3-s bouts, at 5-s intervals, over a 1-min period. As an alternative method of stressing flies, electric shocks were applied across a copper grid at 60 V (direct current) for 1 min. The emitter flies were discarded, the conditioned tube immediately inserted into one side of the T-maze, a fresh tube being inserted on the other side. The elevator containing responder flies was lowered, and the flies given one minute to choose between the two tubes, after which the elevator was partially lifted to block any further choices, and the number of flies in each tube counted. For testing responses to other odorants, 10 µl of 0.01% octanol, 10 µl of 0.01% methylcyclohexanol (MCH) or 10 µl of 0.3 mg ml⁻¹ freshly prepared freeze-dried instant coffee (Taster's Choice) were dripped onto pieces of Whatman filter paper (0.5 × 0.25 in), placed at the end of the 15-ml plastic tube inserted into the T-maze apparatus. The control tube contained filter paper and vehicle alone. The avoidance response was analysed by calculating the PI, that is, the percentage of flies avoiding minus the percentage of flies entering. PI = 0 indicates an equal distribution of flies between the two tubes. PI = 100% indicates that all flies avoided the conditioned tube. Statistical significance was calculated using analysis of variance, ANOVA.

Ca²⁺ imaging and odour delivery

Sample preparation and calcium imaging were as described in ref. 8. CO₂ was diluted to 1% and delivered at a flow rate of 81 ml min⁻¹. dSO and air from non-traumatized flies were prepared as described above, and delivered undiluted.

Gas chromatography and mass spectrometry

Air samples from tubes containing dSO and from fresh tubes were analysed using a gas chromatograph (Agilent 6890) interfaced with a quadrupole mass spectrometer (Agilent 5970B). Five microlitres of air from a tube in which 250 flies had been shaken, or from a fresh tube, were injected with a Hamilton syringe into the GC column at 50°C. The column was then heated to 270°C at a rate of 10°C min⁻¹ with normal inlet temperature of 250°C (splitless mode). The GC column was equipped with a column from J&W, DB5-MS, 30 cm × 0.25 mm (i.d.) × 0.25 µm film thickness. Each molecule eluted from the GC column was detected and its molecular mass and abundance measured by the MS. The operating conditions for the MS were: 10 to 500 *m/z*; 1.64 scans s⁻¹; ionization energy 70 eV.

Respirometer measurements

Emission of carbon dioxide was measured using a Sable System TR-2 carbon dioxide gas respirometry system (Model LI-6251). Groups of 20 flies were placed in a 2.2-ml glass chamber, which was flushed with a constant flow of CO₂-free air through a CO₂ detector. The amount of CO₂ produced by each group of flies was calculated by using DATACAN software (Sable Systems International).

Visualization of murine CD8GFP

Adult fly brains were dissected, fixed in 2% paraformaldehyde, and mounted in Vectashield (Vecta Labs). Native green fluorescence protein (GFP) fluorescence of whole-mount brains was visualized by confocal microscopy (Leica). Olfactory axonal projections of flies bearing GR21A-Gal4 and UAS-mCD8GFP were visualized by fluorescent immunohistochemistry, as described in ref. 16.

Hydroxyurea treatment

The HU protocol⁹ was used to block development of the mushroom body (MB) structure. To check the ablation of the MB, we used the 253Y enhancer trap line carrying UAS-mCD8GFP, which expresses in the MB, as well as in other regions. The survival of the other structures after treatment serves as an internal control for the specificity of HU ablation.

Learning assay

The 'Long Program' training protocol described in ref. 30 was used to train flies. Flies were exposed to 60 s of odour A associated with a 90-V 1.5-s shock delivered every 5 s for 60 s (CS +) followed by 60 s of odour B with no shock (CS -). The trained flies were then given a choice in the T-maze between odours A and B. Both training and testing were done at room temperature (23–25°C) and humidity (20–50%).

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A general mechanism for perceptual decision-making in the human brain

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Findings from single-cell recording studies suggest that a comparison of the outputs of different pools of selectively tuned lower-level sensory neurons may be a general mechanism by which higher-level brain regions compute perceptual decisions. For example, when monkeys must decide whether a noisy field of dots is moving upward or downward, a decision can be formed by computing the difference in responses between lower-level neurons sensitive to upward motion and those sensitive to downward motion^{1–4}. Here we use functional magnetic resonance imaging and a categorization task in which subjects decide whether an image presented is a face or a house to test whether a similar mechanism is also at work for more complex decisions in the human brain and, if so, where in the brain this computation might be performed. Activity within the left dorsolateral prefrontal cortex is greater during easy decisions than during difficult decisions, covaries with the difference signal between face- and house-selective regions in the ventral temporal cortex, and predicts behavioural performance in the categorization task. These findings show that even for complex object categories, the comparison of the outputs of different pools of selectively tuned neurons could be a general mechanism by which the human brain computes perceptual decisions.

Consider driving home from work in clear weather. Stopping at a

light, you see pedestrians waiting to cross the street. Effortlessly, you decide whether one of them is your spouse, your boss or a stranger, and connect the percept with the appropriate action, so that you will either be waving frantically, greeting respectfully or taking another sip of coffee. During a rainstorm, however, the sensory input is noisier, and thus you have to look longer to gather more sensory data to make a decision about the person at the light and the appropriate behavioural response.

This type of decision-making has been studied in single-unit recording studies in monkeys performing sensory discriminations^{5–8}. Shadlen *et al.* proposed that perceptual decisions are made by integrating the difference in spike rates from pools of neurons selectively tuned to different perceptual choices⁹. For example, in a direction-of-motion task, in which the monkey must decide whether a noisy field of dots is moving upward or downward, a decision can be formed by computing the difference in responses between lower-level neurons that are sensitive to upward motion and those sensitive to downward motion^{1–4}. Similarly, in a somatosensory task, in which the monkey must decide which of two vibratory stimuli has a higher frequency, a decision can be formed by subtracting the activities of two populations of sensory neurons that prefer low and high frequencies, respectively^{8,10}. These findings suggest that a comparison of the outputs of different pools of selectively tuned lower-level sensory neurons could be a general mechanism by which higher-level cortical regions compute perceptual decisions^{1,2,11}. However, it is still unknown whether such a mechanism is at work for more complex cognitive operations in the human brain and, if so, where in the brain this computation might be performed.

We used functional magnetic resonance imaging (fMRI) while subjects decided whether an image presented on a screen was a face or a house (Fig. 1). Previous neuroimaging studies have identified regions in the human ventral temporal cortex that are activated more by faces than by houses, and vice versa^{12–16}. Increases in the blood-oxygen-level-dependent (BOLD) signal have been shown to be proportional to changes in neuronal activity in a given region^{17,18}. Therefore larger BOLD responses to faces than to houses and vice versa in specific voxels in the ventral temporal cortex reflect the change in activity in a population of neurons that are more responsive to faces than to houses, and vice versa. Our task thus enabled us to identify two brain regions, one more sensitive to faces and another to houses, and to test whether there are higher-level cortical regions whose output is proportional to the difference in activation in the face- and house-selective regions, respectively.

We based our hypotheses on results from single-unit recording studies in monkeys, which have shown that neuronal activity in areas involved in decision-making gradually increases and then remains elevated until a response is given, with the rate of increase being slower during more difficult trials¹². These studies have also shown that higher-level cortical regions, such as the dorsolateral prefrontal cortex (DLPFC), might form a decision by comparing the output of pools of selectively tuned lower-level sensory neurons^{4,9}. Therefore, we hypothesized that higher-level cortical regions computing a decision would have to fulfil two conditions. First, they should show the greatest activity on trials in which the evidence for a given perceptual category is greatest, for example, a greater fMRI response during decisions about suprathreshold images of faces and houses than during decisions about perithreshold images of these stimuli. Second, their activity should be correlated with the difference between the output signals of the two brain regions containing pools of selectively tuned lower-level sensory neurons involved; that is, those in face- and house-responsive regions.

To test the model of decision-making, we added noise to the face and house stimuli, which made the task arbitrarily more difficult by reducing the sensory evidence available to the subject (Fig. 1b). In the fMRI experiment, subjects viewed images that were either easy (suprathreshold, Fig. 1b top) or difficult (perithreshold, Fig. 1b bottom) to identify as faces or houses.

We identified voxels in the ventral temporal cortex that responded more to faces than to houses, and vice versa, in each subject (Fig. 2a, 'Face' and 'House'). By analogy to the monkey studies, we then used only correct trials to calculate the mean fMRI responses to the four classes of stimuli relative to baseline (fixation) in face- and house-selective regions, respectively. For the preferred category, both face- and house-selective regions responded more to suprathreshold than to perithreshold images whereas the opposite was true for the non-preferred category, indicating that face- and house-selective regions represented the sensory evidence for the two respective categories (Fig. 2b).

Several brain regions typically associated with the attentional network showed a greater response to perithreshold than to suprathreshold stimuli of both faces and houses^{19,20}. These regions, which included the frontal eye field (FEF, Brodmann area (BA)6), the supplementary eye field (SEF) and parietal regions (intraparietal sulcus, IPS), gave a greater response when the task became more difficult, thereby requiring more attentional resources for correct performance (Fig. 3; Supplementary Information).

By contrast, higher-level decision-making areas should show a greater response when decisions are made about suprathreshold images as compared with when decisions are made about perithreshold images of both faces and houses. Several brain regions fulfilled this condition, including a region in the depth of the superior frontal sulcus (BA8/9) within the posterior portion of the DLPFC, the posterior cingulate cortex (BA31) and the superior frontal gyrus (BA9) (Fig. 3). To test the hypothesis that higher-level decision-making areas might use the output from lower-level sensory regions (namely face- and house-selective regions) to form a decision, we first averaged the BOLD signal across voxels in the lower-level face-selective and house-selective regions at each time point. For each participant we thereby derived two time series,

one for the face-selective region and another for the house-selective region (Face(*t*) and House(*t*)). We then computed the absolute difference between these two time series and determined which brain regions covaried with the resulting difference time course ($|Face(t) - House(t)|$). Finally, within these regions we searched for voxels that also showed a greater response during decisions about suprathreshold images relative to decisions about perithreshold images. The only region fulfilling both of the conditions was located in the depth of the left superior frontal sulcus in the posterior portion of the DLPFC (BA8/9, Fig. 4). This region is located just posterior to BA9/46 in the mid-DLPFC and just anterior to the FEF (Fig. 3). Task-related signal changes in the posterior portion of the DLPFC showed a positive correlation with task performance (Fig. 4b).

These results provide strong evidence that perceptual decisions are made by integrating evidence from sensory processing areas.

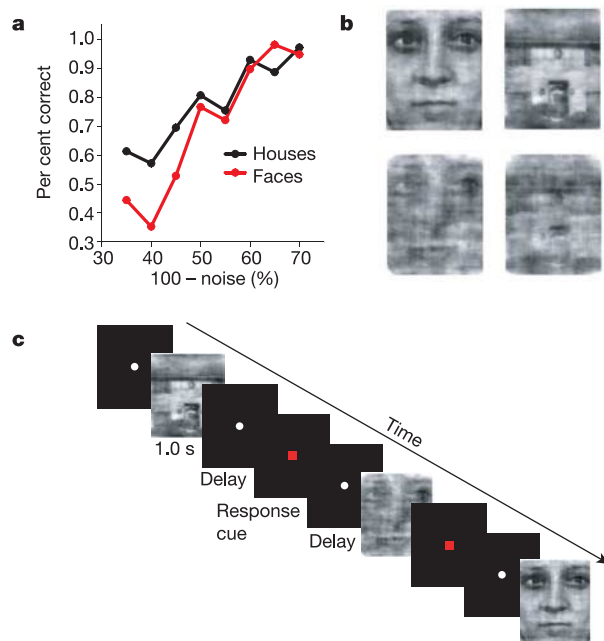


Figure 1 Experimental task. Subjects decided whether an image presented on a screen was a face or a house. By adding noise, the amount of sensory evidence in the stimuli was varied parametrically. **a**, Results of behavioural study to assess the amount of noise to add to the images. Thresholds (82% correct) were about 45% noise for both faces and houses. **b**, In the fMRI experiment, we used images of faces and houses that were either easy (95% correct, suprathreshold, **b** top) or difficult (82% correct, perithreshold, **b** bottom). **c**, Rapid event-related fMRI design. Stimuli were presented for 1 s, subjects responded with a button press after a forced delay (response cue shown for 300 ms, delay 1–5 s).

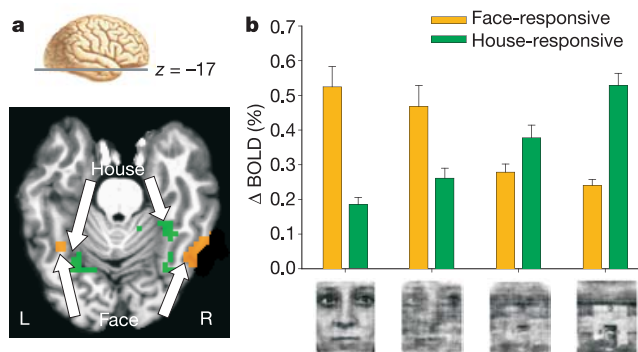


Figure 2 fMRI data illustrating representation of sensory evidence in maximally face- and house-responsive voxels. **a**, Maximally face- (Face, orange) and house-responsive (House, green) voxels in one subject. **b**, BOLD change corresponds to perceptual evidence for respective classes of stimuli. Mean responses ($n = 12$, error bars represent standard error of the mean) in face- and house-selective voxels to the four different conditions (from left to right: suprathreshold face (~10% noise), perithreshold face (~45%), perithreshold house (~53%), suprathreshold house (~10%)). For the respective preferred category, both face- and house-selective regions responded more to suprathreshold than to perithreshold images (face-selective: $P < 0.041$, paired t -test one-tailed; house-selective: $P < 0.001$) while the opposite was true for the non-preferred category (face-selective: $P < 0.013$; house-selective: $P < 0.002$). For face-responsive: suprathreshold face > perithreshold face > perithreshold house > suprathreshold house (analysis of variance, linear contrast, $P < 0.001$); for house-responsive: opposite pattern ($P < 0.001$).

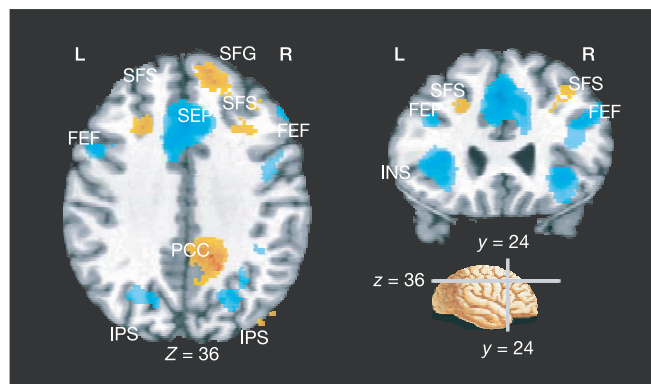


Figure 3 Brain regions showing a main effect of task difficulty: orange: easier (low noise proportion) > harder (high noise proportion); blue: harder > easier. FEF, frontal eye field; INS, insula; IPS, intraparietal sulcus; PCC, posterior cingulate cortex; SEP, supplementary eye field; SFG, superior frontal gyrus; SFS, superior frontal sulcus.

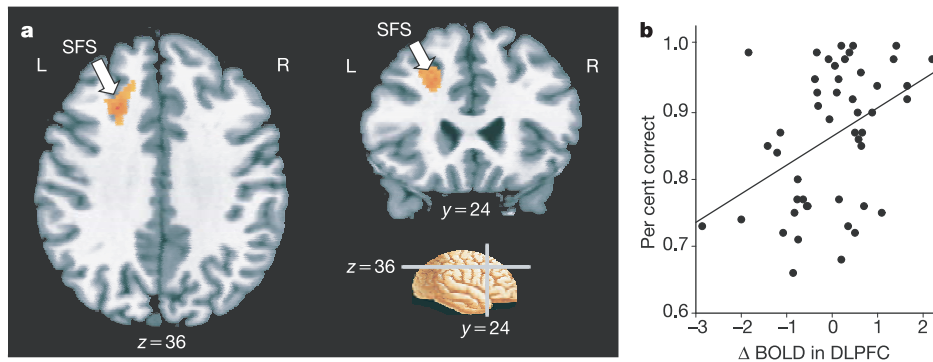


Figure 4 Perceptual decision-making in posterior DLPFC. **a**, Region in the depth of the left SFS, showing both a higher response to suprathreshold images of faces and houses relative to perithreshold images, and a correlation with $|Face(t) - House(t)|$, suggesting that this brain region integrates sensory evidence from sensory processing areas to make a perceptual decision (BA8/9, easier > harder: $x = -24/y = 24/z = 36$, $z_{max} = 4.20$; correlation with $|Face(t) - House(t)|$: $x = -22/y = 26/z = 36$,

$z_{max} = 3.66$, coordinates in MNI system refer to local cluster maxima, and z_{max} to the corresponding z -value). **b**, Signal changes in the posterior portion of the DLPFC predicted task performance ($r = 0.413$, $P = 0.004$). Points represent average BOLD change and performance for each condition (suprathreshold face, perithreshold face, perithreshold house and suprathreshold house) and subject.

Furthermore, our data suggest that in the human brain such a computation might be performed in the left posterior DLPFC. This region fulfilled the two conditions implicit in the Shadlen model of perceptual decision-making^{1,2}. First, the region showed greater activity during those trials in which more sensory evidence for one of the alternative categories was available (suprathreshold versus perithreshold stimuli), and second, the activity in this region was correlated with the absolute difference between the signals of face- and house-selective regions. Finally, signal changes in this region predicted task performance.

Our results using fMRI in humans parallel those using single-unit recordings in monkeys. Recording from a similar region in the posterior DLPFC in monkeys performing a motion discrimination task, Kim and Shadlen² found that neural activity increased proportionally to the strength of the motion signal in the stimulus. Similarly, in our study, the fMRI response was greater when easier decisions were made than when more difficult ones were made. In both monkeys and humans, the outputs of visual processing areas provide the sensory evidence for a decision. In the motion discrimination task, the activity of neurons in cortical area MT, selectively tuned to opposite directions of motion, represent the sensory evidence⁵. Similarly, in our task, the neurons in the ventral temporal cortex tuned to faces and houses, respectively, represent the sensory evidence. Recently, it has been shown in electrical stimulation experiments that the sensory evidence for competing motion directions is compared during the formation of a decision⁴. Similarly, we were able to demonstrate that the sensory evidence for competing categories in maximally face- and house-responsive voxels, respectively, is compared during a decision.

As noted, brain regions in an attentional network played a part in task performance (such as bringing to bear attentional resources), but they did not show the pattern of responses predicted by the model derived from single-unit monkey data. Additionally, control analyses (see Supplementary Information) confirmed that the correlation between BOLD activity in the DLPFC and the difference signal could not be explained either by changes in face- and house-responsive regions alone or by task difficulty, indicating that we have succeeded in localizing the subtraction operation to the left posterior DLPFC.

There is evidence that the involvement of the DLPFC in decision-making processes is not specific to our task but that it generalizes across different tasks. For example, the same area in the left posterior DLPFC that we observed in our study was activated in a positron-emission tomography (PET) study when subjects per-

formed a visual conditional task, such as 'if you see a red cue, point to the pattern with stripes, but if you see a blue cue point to the pattern with red circles'²¹ (see also ref. 22). Moreover, we found that when subjects performed the same direction-of-motion discrimination task that was used in the single-unit recording studies, the identical left posterior DLPFC showed a greater fMRI response to stronger motion signals, whether the subjects responded with a button press or an eye movement³¹. These studies thus indicate that this prefrontal region has general decision-making functions, independent of stimulus and response modalities. Our results are also consistent with reports that lesions in the posterior DLPFC impair conditional discrimination tasks in both monkeys and humans^{21,23}. Others have suggested that the main function of the prefrontal cortex is to guide activity along task-relevant pathways from lower-level sensory regions to areas that plan and execute responses²⁴. Here, we have demonstrated a mechanism for how perceptual decision-making processes might be instantiated in the human brain, using a relatively simple subtraction mechanism. What remains to be shown is how this model can account for additional variables that affect the decision-making process, such as the expected value of different options²⁵, the prior probability of the appearance of different options²⁶ and their internal valuation²⁷. Ideally, this model and the mechanisms described here will also help to explain the much more complicated decisions we confront in everyday life. □

Methods

Subjects

Twelve healthy volunteers participated in the imaging experiment (6 females, mean age 31.1) and 12 healthy volunteers in the behavioural experiment (7 females, mean age 26.8). All were right-handed, had normal or corrected vision, no past neurological or psychiatric history and no structural brain abnormality. Informed consent was obtained according to procedures approved by the NIMH-IRP Internal Review Board.

Visual stimuli

A set of 38 images of faces (face database, MPI for Biological Cybernetics, Germany) and houses were used. Fast fourier transforms (FFT) of these images were computed, producing 38 magnitude and 38 phase matrices. The average magnitude matrix of this set was stored, then stimulus images were produced by calculating the inverse FFT (IFFT) of the average magnitude matrix and individual phase matrices. The phase matrix used for the IFFT was a linear combination of the original phase matrix computed during the forward fourier transform and a random noise matrix. The resulting images all had an identical frequency power spectrum²⁸ (corresponding to the average magnitude matrix) with graded amounts of noise.

Task

Images of faces and houses with low (per cent correct above 95%) and high (corresponding to the 82%-threshold) proportions of noise, respectively (see Fig. 1b) were

presented using the Psychophysics Toolbox (www.psychtoolbox.org) under Matlab (Mathworks). Stimuli were projected for 1 s onto a back-projection screen using an LCD projector (Sharp). Subjects decided whether an image was a face or a house and responded with a button press after a forced delay, which was included to make the task as analogous to the studies in monkeys as possible (those experiments typically include a forced delay¹) (compare Fig. 1c). The sequence of events was optimized using rsfgen (AFNI, <http://afni.nimh.nih.gov>).

Data acquisition and analysis

Whole-brain MRI data were collected on a 3T GE Signa (GE Medical Systems). Echoplanar data were acquired using standard parameters (Field of view, 200 mm; matrix, 64 × 64; 25 axial slices, 5 mm thick; in-plane resolution, 3.125 mm; repetition time, TR, 2.0 s; error time, TE, 30 ms; flip angle, 90°). Five to eight runs of 162 volumes each were acquired. The first four volumes were discarded to allow for magnetization equilibration. To minimize head motion, we used both a bite bar and a vacuum head pad. AT1 weighted volume (MP-RAGE) was acquired for anatomical comparison.

MRI data were analysed using a mixed effects approach within the framework of the general linear model (GLM as implemented in FSL 5.0, <http://www.fmrib.ox.ac.uk/fsl>). Pre-processing was applied: slice-time correction, motion correction, non-brain removal, spatial smoothing using a kernel of 8 mm full-width at half-maximum, mean-based intensity normalization of all volumes by the same factor; highpass temporal filtering (gaussian-weighted least-squares straight line fitting, with sigma = 50.0 s). Time-series statistical analysis was carried out using FSL with local autocorrelation correction²⁹. Trials in which subjects gave no response or an incorrect response were pooled together and modelled as a regressor of no interest (error trials). Hence, similar to the studies in monkeys, only correct trials were used to model regressors for the four conditions (suprathreshold face, perithreshold face, perithreshold house and suprathreshold house). The average number of trials was 215.5 ± 29.7 (mean ± s.d.) for suprathreshold face, 190.17 ± 31.6 for perithreshold face, 194.7 ± 30.8 for perithreshold house and 202.8 ± 26.8 for suprathreshold house, respectively. Time series were modelled using event-related regressors for each of the four conditions as well as error trials, and convolved with the haemodynamic response function (gamma variate). Contrast images for each condition and the contrasts of interest for each subject were computed and transformed, after spatial normalization, into standard (MNI152) space. Group effects were computed using the transformed contrast images in a mixed effects model treating subjects as random. The resulting Z statistic images were thresholded at Z > 3.1, corresponding to P < 0.001, uncorrected. For display purposes, statistic images are shown with Z > 2.6, corresponding to P < 0.005. In each subject we determined voxels in the temporal cortex that were more responsive to faces than to houses, and vice versa. The region analysed included the lingual, parahippocampal, fusiform and inferior temporal gyri 70 to 20 mm posterior to the anterior commissure in Talairach brain atlas coordinates (see ref. 30). To test in which voxels the BOLD signal significantly covaried with |Face(t) − House(t)|, we set up an additional model using the following three regressors: (1) the difference between the regressors for the suprathreshold and the perithreshold conditions used in the GLM analysis described above ((suprathreshold face + suprathreshold house) − (perithreshold face + perithreshold house)); (2) the absolute difference between the time series in face- and house-responsive voxels in each subject (|Face(t) − House(t)|); and (3) the product of the first and second regressors, representing the interaction between (|Face(t) − House(t)|) (physiological signal) and task-related parameters (psychological factor, hence psychophysiological interaction). Contrast images and group effects were computed as described above. We did not find any voxels in which changes in activity significantly covaried with the psychophysiological interaction term.

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Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*

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Daily rhythms of physiology and behaviour are precisely timed by an endogenous circadian clock^{1,2}. These include separate bouts of morning and evening activity, characteristic of *Drosophila melanogaster* and many other taxa, including mammals^{3–5}. Whereas multiple oscillators have long been proposed to orchestrate such complex behavioural programmes⁶, their nature and interplay have remained elusive. By using cell-specific ablation, we show that the timing of morning and evening activity in

Drosophila derives from two distinct groups of circadian neurons: morning activity from the ventral lateral neurons that express the neuropeptide PDF, and evening activity from another group of cells, including the dorsal lateral neurons. Although the two oscillators can function autonomously, cell-specific rescue experiments with circadian clock mutants indicate that they are functionally coupled.

Circadian rhythms arose during evolution, by adapting to and then anticipating the 24-h rotation of the Earth and its consequent light/dark cycle^{1,2}. During a generic day of 12 h of daylight followed by 12 h of darkness (LD), wild-type *D. melanogaster* flies exhibit two distinct bouts of locomotor activity: one is centred at dawn (morning) and the other at dusk (evening), similar to many crepuscular insect species. The morning and evening activity peaks begin before lights-on and lights-off, respectively, indicating that flies anticipate the discontinuous morning (dark to light) and evening (light to dark) transitions that take place under LD conditions. Although there are some indications that the morning peak may not require all of the canonical oscillator components^{3,7}, there is other evidence that the morning as well as the evening anticipation requires an endogenous circadian oscillator^{4,5,7}. For

example, a number of arrhythmic mutants fail to ramp-up their activity during the last 2–3 h of daytime and night time; instead they exhibit driven periodic behaviour, consisting of abrupt activity elevations (startle responses) immediately after lights-on and lights-off⁹.

There are approximately 100 circadian clock neurons, bilaterally clustered in six defined groups of cells within the adult fly brain⁸. Two of these regions, the small and large ventral lateral neurons (LN_vs), express the neuropeptide PDF (also referred to hereafter as PDF⁺ or PDF-expressing cells) and make an important contribution to circadian locomotor activity rhythms^{9–11}. Ablation of these cells by targeted expression of proapoptotic genes causes arrhythmicity in DD conditions¹⁰. Our recent study indicated that the LN_vs are key cellular coordinators that synchronize or maintain rhythmic gene expression within the circadian network¹². However, ablation of the LN_vs affects only limited aspects of behavioural rhythms under LD conditions¹⁰, indicating that there are other important cellular substrates that control circadian behaviour under natural conditions.

To identify these additional circadian clock cells, we sought to assay locomotor activity in a strain ablated of more clock neurons

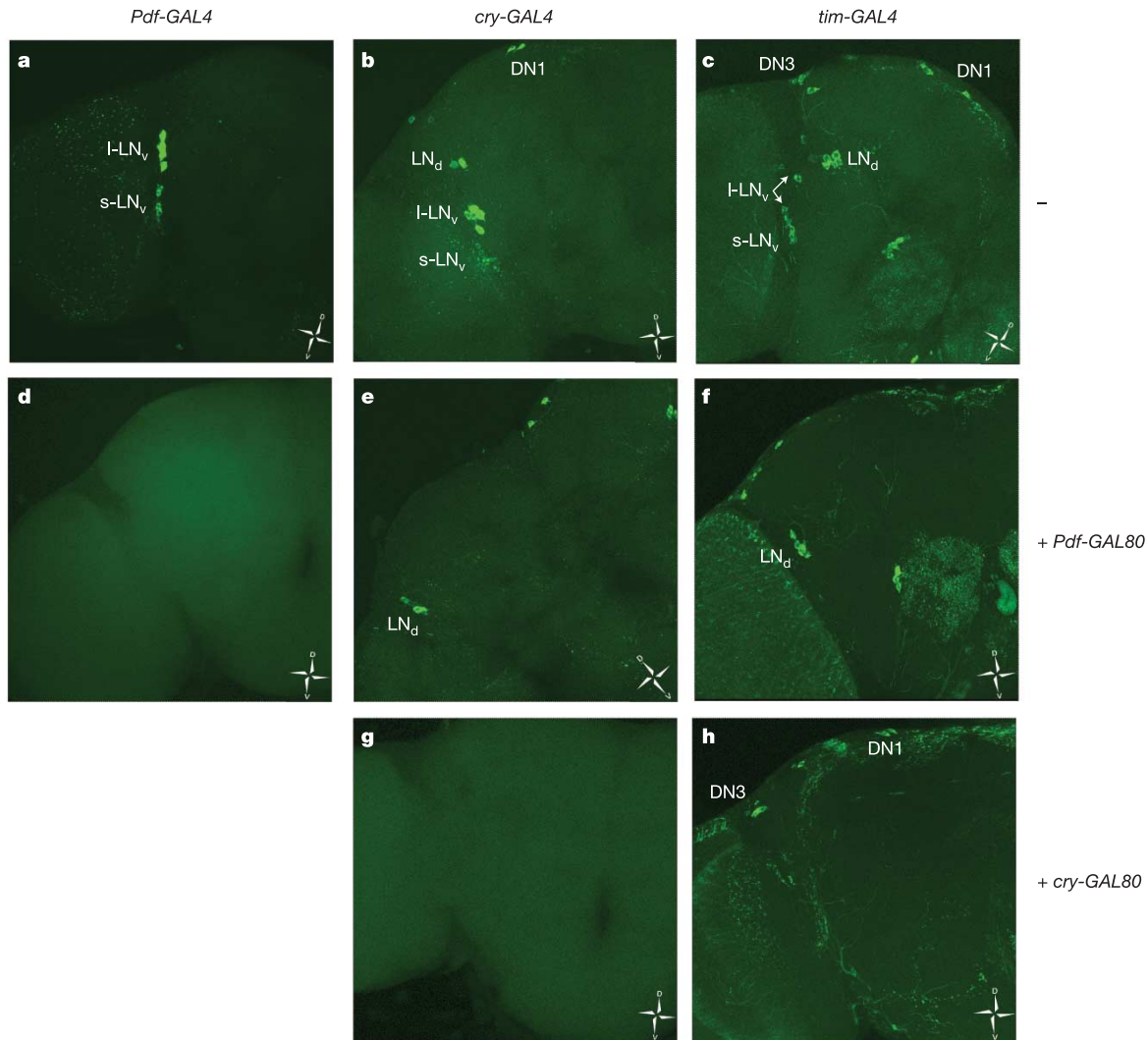


Figure 1 Suppression of *GAL4* activity with *GAL80* in *Drosophila* circadian neurons, as examined with a *UAS-ANFGFP* reporter. **a–c**, *GAL4* only. **a**, *Pdf-GAL4*-targeted expression in LN_vs¹¹. **b**, *cry-GAL4* in LN_vs, LN_ds and two DN1 cells^{13–15}. **c**, *tim-GAL4* (ref. 8) in all circadian neuronal groups. **d–f**, *Pdf-GAL80* suppresses *GAL4* activation in the LN_vs. **d**, *Pdf-GAL4*; *Pdf-GAL80*; *UAS-ANFGFP*. **e**, *cry-GAL4*; *Pdf-GAL80*; *UAS-*

ANFGFP. **f**, *tim-GAL4*; *Pdf-GAL80*; *UAS-ANFGFP*. **g**, *cry-GAL80* suppresses *GAL4* in a wider group of neurons. **g**, *cry-GAL4*; *cry-GAL80*; *UAS-ANFGFP*. **h**, *tim-GAL4*; *cry-GAL80*; *UAS-ANFGFP*. In each panel a compass rose indicates the orientation of the brain (D, dorsal; V, ventral). See Supplementary Table S1 for further details.

than just the LN_v s. The *tim-GAL4* transgene drives gene expression ubiquitously in all six groups of clock cells⁸; that is, in the three groups of dorsal neurons (DN1, DN2, DN3), the dorsal-lateral neurons (LN_d s) as well as the two groups of PDF-expressing LN_v s.

However, targeted expression of the proapoptotic gene *hid* with the *tim-GAL4* driver led to embryonic lethality, precluding any assay of locomotor activity. The circadian photoreceptor *cryptochrome* (*cry*) gene is expressed in many circadian neurons of the adult brain^{13,14},

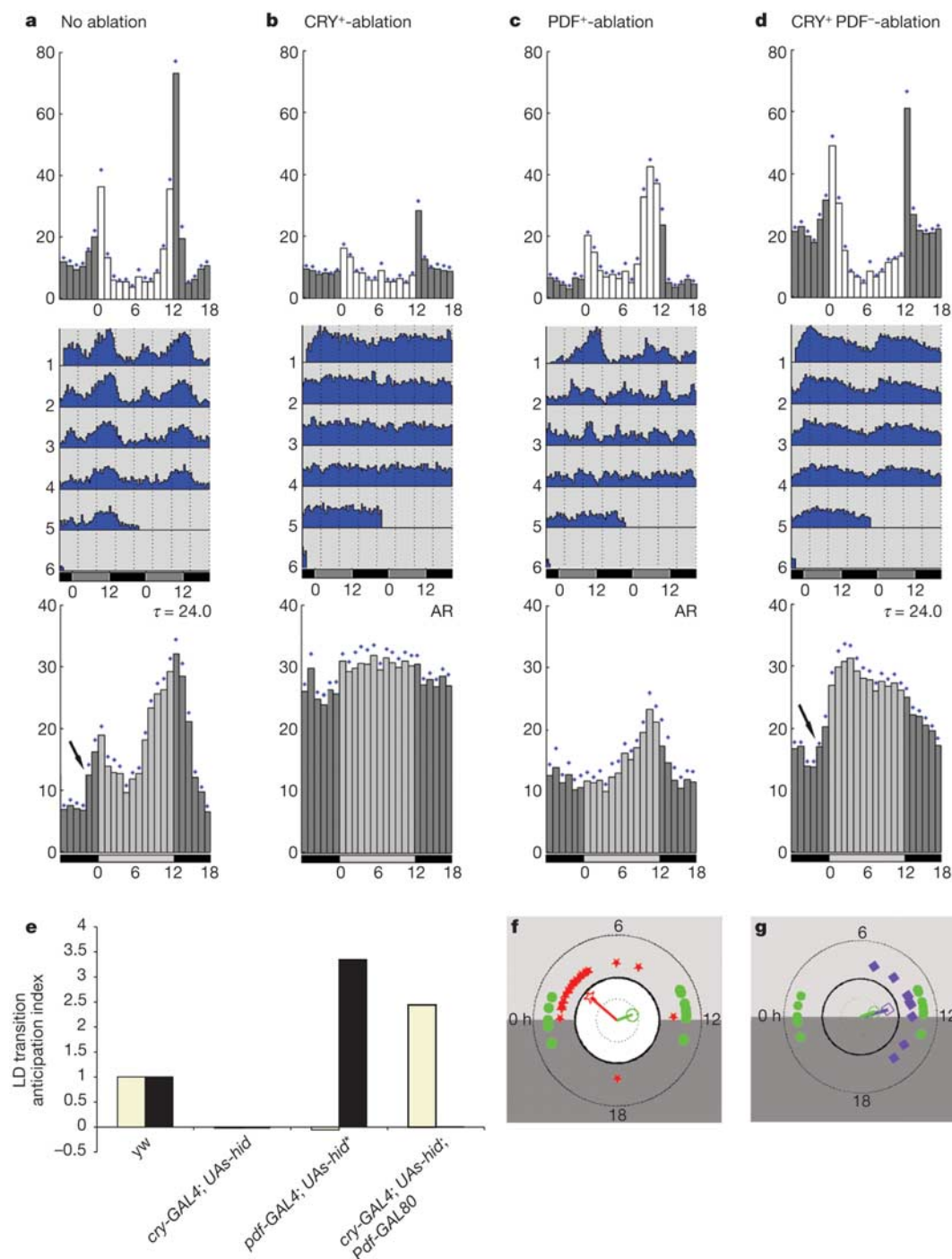


Figure 2 Genetic ablation of different groups of circadian neurons causes the loss of different locomotor activity components. **a–d**, Comparison of the circadian locomotor behaviour of normal rhythm (*y w*) flies (**a**) with that of transgenic flies with different circadian neurons ablated (**b–d**). Top panels, group LD activity in histogram format; middle panels, double-plotted actograms of group DD activity; bottom panels, the same group DD activities plotted as histograms. In the histograms, the lighting condition is indicated by the colour of the activity bin (LD: white = day and dark grey = night; DD: light grey = subjective day and dark grey = subjective night). The blue diamonds indicate the standard error of the mean. **a**, *y w* flies; **b**, [*CRY⁺*]-ablated flies (*cry-GAL4; UAS-hid*); **c**, [*PDF⁺*]-ablated flies (*pdf-GAL4; UAS-hid*); **d**, [*CRY⁺PDF⁺*]-

ablated flies (*cry-GAL4; Pdf-GAL80; UAS-hid*). **e**, Summary of LD lights-on and lights-off anticipation for wild type and for the three ablation genotypes (see Methods for the normalized AI). Asterisk, evening anticipation of [*PDF⁺*]-ablated flies, shifted 1 h¹⁰. Yellow columns indicate mornings and black columns indicate evenings. **f**, **g**, Circular phase analysis of DD locomotor activity for individual flies. The green circles represent phase estimates for *y w* flies. **f**, [*CRY⁺PDF⁺*]-ablated flies (*cry-GAL4; Pdf-GAL80; UAS-hid*; red stars). **g**, [*PDF⁺*]-ablated flies (*pdf-GAL4; UAS-hid*; blue squares). The timing is shown in CT hours. Subjective day (CT0–12) is in light grey whereas subjective night (CT12–24) is dark grey. See Supplementary Table S4 for further details.

much more broadly than *Pdf*. On the basis of the supposition that these light-sensing neurons may have a prominent role in controlling LD rhythms, we focused on a previously characterized *cry-GAL4* driver (*cry₁₃-GAL4*)¹³ that induces expression in the LN_v and LN_d cells (Fig. 1b)¹³. To clarify an inconsistency in the previous description of this driver¹⁵, we used a stronger green fluorescent protein (GFP) reporter¹⁶. The large LN_vs were the most prominently stained cells, and all lateral neurons (that is, LN_vs and LN_ds) were GFP-positive. We also observed consistent GFP expression in two additional locations: two very dorsal DN1 cells (Fig. 1b, e) and the one PDF-negative LN_v cell¹⁷ (Supplementary Table S1). The most dorsally positioned pair of adult DN1 cells was recently shown to express high levels of CRY, lack expression of Glass protein and correspond to the two larval DN1 cells¹⁴. Although CRY is probably even more widely expressed¹⁴, we interpret this pattern of LN_vs, LN_ds and 2 DN1 cells to reflect circadian neurons with particularly high *cry* expression, and will refer to these cells as CRY⁺.

The pattern of LD locomotor activity for *cry-GAL4; UAS-hid* flies (which are viable and morphologically normal) was conspicuously different from that of wild-type (Fig. 2a, top panel) and of LN_v-ablated flies (*Pdf-GAL4; UAS-hid*, Fig. 2c, top panel) and is one of the most severe circadian defects known so far: there is no anticipation of either lights-on or lights-off (Fig. 2b, top panel). In DD, the *cry-GAL4; UAS-hid* strain is also completely arrhythmic (Fig. 2b, middle and bottom panels). Although this does not exclude an important role for the dorsal neurons in persistent oscillator function and entrainment^{14,18}, most of the dorsal neurons (which survive the ablation; Fig. 3b) are apparently incapable of driving circadian behaviour alone. We also hypothesized that the marked difference between the *cry-GAL4* ablation and the *Pdf-GAL4* ablation is due to the additional CRY-expressing cells in the former (that is, at least the LN_ds, the two DN1 cells and the PDF[−] LN_v). We will henceforth refer to these cells collectively as CRY⁺PDF[−].

To study the function of the CRY⁺PDF[−] cells and their interaction with the PDF-expressing cells, we developed a novel ternary expression system to refine the spatial resolution of clock cell targeting. On the basis of the MARCM system¹⁹, we generated transgenic strains that express *GAL80* under the control of the *Pdf* and *cry* promoters^{11,13}. To assay the suppression of *GAL4*-mediated transcriptional activation, we crossed the *GAL80* strains to flies expressing GFP in specific clock cells, under various *GAL4* drivers. *Pdf-GAL4; UAS-ANFGFP; Pdf-GAL80* and *cry-GAL4; UAS-ANFGFP; cry-GAL80* have no detectable GFP signal in the brain, suggesting that the repressive activities of *Pdf-GAL80* and *cry-GAL80* are sufficient to counteract the transcriptional activity of their respective *GAL4* drivers (Fig. 1d, g; see also Supplementary Table S1). When the *cry-GAL4* driver is combined with *Pdf-GAL80* repressor (*cry-GAL4; UAS-ANFGFP; Pdf-GAL80*, Fig. 1e; see also Supplementary Table S1), consistent GFP signal is restricted to the LN_ds, the two DN1 cells and one LN_v cell (probably the PDF[−] s-LN_v cell); this is as predicted by the difference in the two promoters (Fig. 1a, b). When the pan-clock-cell *tim-GAL4* driver is combined with the *Pdf-GAL80* repressor, the expression pattern recapitulates the described *tim-GAL4* pattern with the exception of the LN_vs (Fig. 1f). The *tim-GAL4; cry-GAL80* combination excludes GFP signal from the LN_ds and the LN_vs (Fig. 1h; see also Supplementary Table S1).

We also assayed the efficiency of *GAL80* suppression through the use of a behavioural readout. In both cases, the *GAL80* transgenes fully rescued the behavioural defect caused by the corresponding *GAL4*-mediated cell ablation (Supplementary Tables S2 and S3). As an extra precaution, we chose two of the most potent lines expressing *Pdf-GAL80* and *cry-GAL80* and generated double insert recombinants (*Pdf-GAL80_{96A}* and *cry-GAL80_{2e3m}*, respectively; Supplementary Tables S2 and S3), which were used in all subsequent experiments.

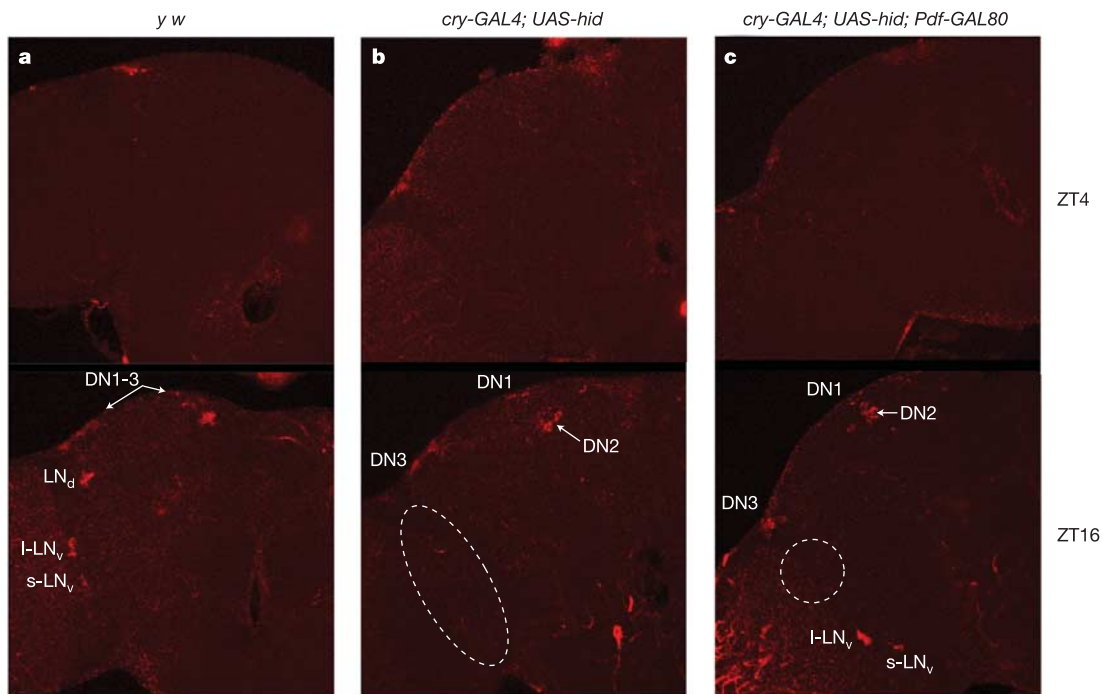


Figure 3 Targeted genetic ablation removes specific circadian neurons. Cell-specific ablation in the brain was confirmed by whole-mount *in situ* hybridization with a *tim* probe. **a**, In *yw* flies, all circadian neurons show robust *tim* oscillation between ZT4 (top) and ZT16 (bottom). ZT, Zeitgeber time, defines the timing of the LD experiment: ZT0 = the time of lights on, whereas ZT12 = the time of the lights off event. **b**, In *cry-GAL4; UAS-hid*

flies, both LN_v and LN_d neurons are missing (dashed circles), whereas most DN cells survive the ablation and oscillate indistinguishably from wild type. **c**, In *cry-GAL4; Pdf-GAL80; UAS-hid* flies, the LN_d neurons are missing (dashed circles), whereas the LN_vs and DN cells oscillate like wild type. See 'Imaging techniques' in Methods for details.

To ablate specifically the CRY^+PDF^- cells, we crossed *Pdf-GAL80* with *cry-GAL4; UAS-hid* and generated progeny carrying all three transgenes (*cry-GAL4; Pdf-GAL80; UAS-hid*). *tim in situ* hybridizations confirmed the specific ablation of these cells (Fig. 3c). This triple transgenic strain allowed a behavioural comparison of the ablation of these three sets of neurons: PDF^+ , CRY^+PDF^- and CRY^+ (Fig. 2; see also Supplementary Table S4).

The LD behaviour of the $[CRY^+PDF^-]$ -ablated flies (Fig. 2d, top) appeared to be intermediate between the complete loss-of-rhythm phenotype of the $[CRY^+]$ -ablated flies (Fig. 2b, top) and the largely normal phenotype of the $[PDF^+]$ -ablated flies (Fig. 2c, top). On closer inspection, however, we noticed that the $[CRY^+PDF^-]$ -ablated flies showed suppressed evening activity before lights-off (Fig. 2d, top panel, and e). The residual evening peak occurs after

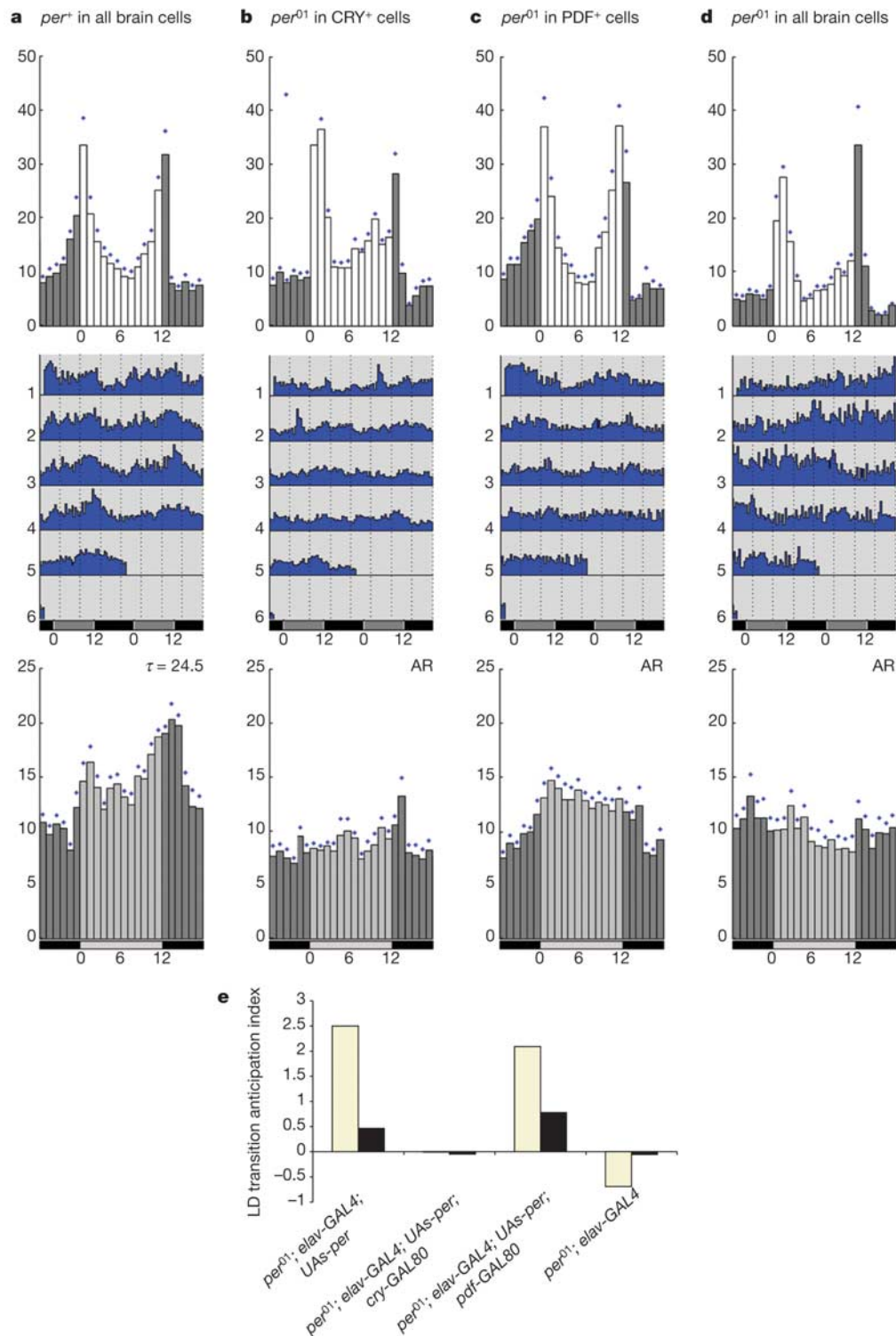


Figure 4 Functional disruption of the two oscillators. The panels are as in Fig. 2. **a**, *per⁰¹* rescue in all of the brain neurons (*per⁰¹; elav-GAL4; UAS-per*). **b**, Suppression of rescue in CRY^+ neurons (*per⁰¹; elav-GAL4; UAS-per; cry-GAL80*). **c**, Suppression of rescue in

PDF neurons (*per⁰¹; elav-GAL4; UAS-per2-4; Pdf-GAL80*). **d**, Non-rescued *per⁰¹* control (*per⁰¹; elav-GAL4*). **e**, Group anticipation indices for the genotypes analysed in **a–d** (as in Fig. 2e). See Supplementary Table S5 for further details.

lights-off and almost certainly reflects an evening startle response, with little or no bona fide circadian anticipation³. In contrast, there was a robust morning peak with normal circadian anticipation of lights-on (Fig. 2d, top panel, and e). The absence of lights-off anticipation was reminiscent of the diminished morning activity peak reported in [PDF⁺]-ablated flies or in the *Pdf⁰¹* (PDF-null) mutant strain¹⁰. We checked the LD activity pattern of these genotypes and confirmed that they lack lights-on anticipation (Fig. 2c, e, and data not shown). The results indicate that the CRY⁺PDF⁻ cells govern the evening activity peak, whereas the PDF-expressing cells govern the morning activity peak; these two oscillators can function independently.

The behaviour of these strains in DD does not differ from LD, which verifies our conclusions and confirms that neither activity peak is driven by light (Fig. 2a–d, middle and bottom panels). In contrast to the [PDF⁺]-ablation (in which the locomotor activity rhythm damps down during the first few days in DD; Fig. 2c, middle panel) and the [CRY⁺]-ablation (which abolishes rhythmicity; Fig. 2b), the [CRY⁺PDF⁻]-ablation is characterized by a robust and sustained rhythm in DD with a wild-type period ($\tau = 24.0$ h; Fig. 2d). However, these [CRY⁺PDF⁻]-ablated flies have a unimodal, apparently morning-only activity pattern (Fig. 2d, bottom). The timing of the morning locomotor activity increase is indistinguishable from that of wild type (Fig. 2a, d, arrows), but [CRY⁺PDF⁻]-ablated flies fail to suppress strongly the activity after subjective morning. This prolonged activity bout delays the calculated phase by more than 2 h relative to the phase of wild-type morning activity (Fig. 2f; see also Supplementary Table S4)³. The absence of a robust DD evening activity bout in the [CRY⁺PDF⁻]-ablated flies confirms the importance of these cells for this aspect of locomotor output (Fig. 2d). [PDF⁺]-ablated flies as well as the *Pdf⁰¹* mutant strain also have a predominantly unimodal locomotor activity pattern that corresponds to subjective evening¹⁰ (Fig. 2c, g, and data not shown). Although these flies are only weakly

rhythmic during the first few days of DD, the absence of a morning peak is evident¹⁰ (Fig. 2e, g) and confirms the importance of the PDF-expressing cells for this aspect of the activity programme. We propose that these two oscillators generate distinct output signals to govern the morning and evening activity peaks, respectively. Because the *Pdf⁰¹* mutant is missing the morning peak yet retains intact transcriptional oscillations in the LN_vs under LD conditions¹², the PDF peptide is a strong candidate for the morning output signal from the LN_vs.

The notion of differentially timed outputs is based on the synchrony of the circadian programmes within the CRY⁺ cells; that is, the indistinguishable phase of molecular oscillation within all clock neurons in the brain as indicated by *in situ* hybridization^{12,15} or immunohistochemistry⁸. On the basis of our recent evidence indicating that clock cells constitute a network¹², we used another strategy to examine the autonomy of the CRY⁺PDF⁻ and PDF⁺ cells and selectively disrupted their molecular oscillations. *per⁰¹* is a canonical circadian mutant that eliminates the Period protein (PER) and causes the loss of both molecular and behavioural rhythmicity²⁰. A *UAS-per* transgene (*UAS-per2-4*), driven by the pan-neuronal *elav-GAL4* driver (*elav^{C155}-GAL4*), was previously used to rescue the behavioural defect of *per⁰¹* (ref. 21). We verified this result (Fig. 4a; see also Supplementary Table S5) and then introduced *cry-GAL80* into this system to block rescue in the CRY⁺ cells. The resulting genotype (*per⁰¹; elav-GAL4; UAS-per; cry-GAL80*) was completely arrhythmic (AR) in DD (Fig. 4b, bottom panel) and in LD (Fig. 4b, top panel, and e) conditions. This mimics the behavioural phenotype of [CRY⁺]-ablated flies and verifies that both activity peaks are predominantly clock-regulated. We also introduced *Pdf-GAL80* instead of *cry-GAL80* into the system to block rescue of *per⁰¹* only in the PDF-expressing cells (*per⁰¹; elav-GAL4; UAS-per; Pdf-GAL80*). The block was effective because the flies became gradually arrhythmic under DD conditions (Fig. 4c, middle and bottom panels), and mimicked the phenotype of [PDF⁺]-ablated or *Pdf⁰¹* flies¹⁰.

The LD behaviour of these LN_v-*per⁰¹* flies, however, was unexpected: they manifested a wild-type-like anticipation of lights-on as well as lights-off (Fig. 4c, f), as opposed to [PDF⁺]-ablated flies, which only have a lights-off (evening) peak in LD (Fig. 2e). As anticipation of morning is a characteristic of a functional LN_v clock, we interpret the lights-on peak to indicate the recovery of at least PDF output function. As the *cry-GAL80*-blocked flies are arrhythmic and show essentially no anticipation (Fig. 4c, e), this recovery of a morning peak requires a functional oscillator within the CRY⁺PDF⁻ cells. These evening peak CRY⁺PDF⁻ cells can apparently drive the clockless PDF neurons to direct a seemingly normal morning peak under LD conditions.

Pittendrigh and Daan proposed the existence of two circadian oscillators to explain the morning and evening activity peaks characteristic of diurnal behaviour in many species⁶. Our genetic dissection of the *Drosophila* circadian system shows that there are indeed two oscillators, which are located in two distinct cell groups. They can act independently and control these two canonical features of the wild-type behavioural programme: the PDF⁺ cells drive the morning activity peak, and the CRY⁺PDF⁻ neurons drive the evening peak (Fig. 5). The latter may be specialized daytime cells, because the evening peak begins in the middle of the light period. Similarly, the PDF⁺ cells may be night time cells. This also fits with the importance of the PDF-expressing cells for rhythmicity in constant darkness^{10,12}.

Although a comparable functional dissection of the mammalian circadian system has not been reported, different portions of the suprachiasmatic nuclei (SCN) probably control different aspects of the circadian programme^{22,23}. Indeed, the left and right SCN may constitute separable oscillators²⁴. Recent results also show that clocks in different SCN subregions have different phases²⁵. Although this possibility needs to be examined more carefully in

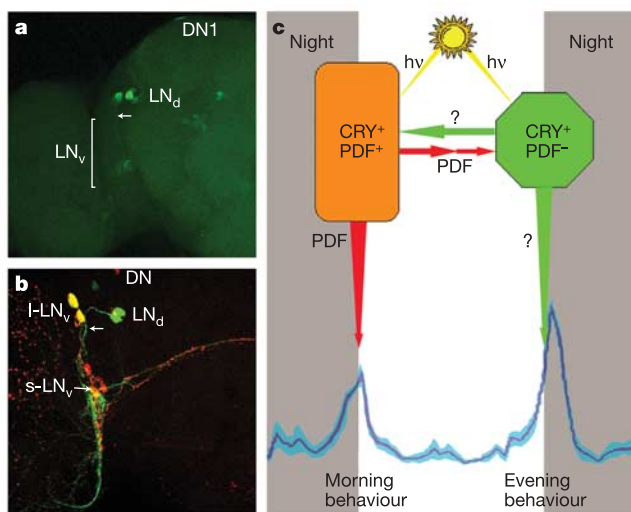


Figure 5 Coupling of the two *Drosophila* oscillators CRY⁺PDF⁻ and PDF⁺ underlies the coordination of the two daily activity peaks. **a**, CRY⁺PDF⁻ cells project neurites onto the LN_v region, visualized here with a membrane-labelling GFP reporter (green; *cry-GAL4; Pdf-GAL80; UAS-mCD8iGFP*). **b**, PDF immunostaining (red) of *cry-GAL4; UAS-mCD8iGFP* adult brains shows that CRY⁺PDF⁻ cells send a PDF⁺ axonal process (green, arrow) towards the region of PDF⁺ cells (LN_vs, in red). The red dots localized in the LN_d and DN regions might indicate a reciprocal connection (PDF peptide released from LN_v terminals). **c**, Model of the circadian network responsible for morning and evening activity in *Drosophila*. PDF⁺ and CRY⁺PDF⁻ cells function as morning and evening oscillators, respectively. A wild-type diel activity plot is shown in blue. hv indicates the action of light.

flies, there is at present no evidence for significant phase differences between the six groups of adult brain neurons. This is consistent with the notion that the morning and evening outputs from the two relevant cell groups connect independently to the same core oscillator mechanism. Although we cannot functionally distinguish between the few CRY^+PDF^- cells (that is, the LN_d s, the two $DN1$ cells and the single PDF^- small LN_v) or between the more weakly expressing clock cells, we favour the LN_d s as the evening cell group, because of efferent projection from the LN_d s to the LN_v region (Fig. 5a, b). Finally, recent evidence suggests that LN_v s on the one hand and some more dorsal neurons (LN_d s and some DN cells) on the other can constitute distinct oscillators under some conditions²⁶.

Functional restoration of the morning activity peak in *per*⁰¹ PDF^+ cells could occur by driving oscillator function or by driving the output without rescuing the intracellular oscillator itself. Although previous work has suggested that intercellular communication can help maintain robust intracellular oscillations in both flies and mammals^{12,25,27,28}, similar studies have not been done in the context of a defective oscillator. It will therefore be important to determine which aspects of the molecular programme can be externally driven in the absence of a key endogenous clock component. In any case, it is likely that coupling of the two *Drosophila* oscillators coordinates the two major activity peaks and facilitates a response to environmental challenges such as seasonal changes. Complex intercellular regulation probably accounts for the evolution of even more complicated circadian systems such as the mammalian SCN.

Note added in proof: While this manuscript was in review, we learned that F. Rouyer and colleagues³¹ had carried out similar experiments and reached almost identical conclusions, namely that the LN_d s drive the evening activity peak and the LN_v s the morning peak. □

Methods

Drosophila genetics

The *GAL80* open reading frame and SV40 poly A sequence was amplified by polymerase chain reaction (PCR) from tubulinP-*GAL80* (ref. 19) and subcloned in pBluescript SK vectors under *Pdf* 2.4-kilobase (kb)¹¹ and *cry* 5.5-kb¹³ promoters, respectively. The fused *Pdf-GAL80* and *cry-GAL80* constructs were excised and cloned into pCaSpeR3 vector between *Sac*II and *Xho*I (*Pdf-GAL80*), and *Bam*HI and *Xho*I (*cry-GAL80*). The transformation plasmids were used to generate transgenic flies through standard embryo microinjection procedures. Eleven *Pdf-GAL80* and twenty *cry-GAL80* lines were obtained (Supplementary Tables S2 and S3). Recombinant chromosomes bearing multiple copies of each transgene were generated and verified for their ability to suppress *GAL4* transcription. In all the presented experiments, the *GAL80* strains used (*Pdf-GAL80*^{96A} and *cry-GAL80*^{2c3m}) carried two inserts of the respective *GAL80* transgenes. *UAS-per2-4* transgenic flies²¹ were provided by A. Sehgal and *UAS-ANFGFP* (ref. 16) flies by E. Levitan.

Behavioural analysis

Flies were entrained for 3 days in 12 h light/12 h dark (LD) conditions before initiating an LD experiment and for at least 5 days for each DD experiment. Locomotor activities of individual flies were monitored using Trikinetics *Drosophila* activity monitors. The analysis was done with a signal processing toolbox²⁹ implemented in MATLAB (MathWorks). Autocorrelation and spectral analysis were used to assess rhythmicity and to estimate period. The phase information was obtained by using circular statistics³⁰. A low-pass butterworth filter was applied to detect the less robust subjective morning peak of wild-type flies. The anticipation index ($AI = b_{-1}(b_{-1} - b_{-2})(b_{-2} - b_{-3})/b_{+1}$) was developed to reflect the fact that anticipation of light transition is directly proportional to the gradual increase in activity before the transition ($(b_{-1} - b_{-2})(b_{-2} - b_{-3})$) and inversely proportional to the startle effect (b_{+1}/b_{-1}), where b_i = activity counts in bin i , and i = number of bin before (− i) and after (+ i) light switch. Finally, the morning and evening anticipation index of each genotype was normalized to that of wild type ($AI_{\text{WT}} = 1$). Other methods of measuring the anticipation gave similar results (data not shown).

Imaging techniques

Fly brains were dissected and mounted as previously described¹². *In situ* hybridization of *tim* has also been described¹⁵. At least 15 brains were examined for each time and genotype in two independent experiments, with indistinguishable results (Fig. 3). The *in situ* experiment shown in Fig. 3c was carried out four independent times, with identical results. For immunodetection of PDF, adult fly brains were incubated with rabbit anti-PDF (dilution 1:5,000). Donkey anti-rabbit IgG (TexasRed-conjugated, Jackson

ImmunoResearch) was used at 1:200 dilution. A Leica laser-scanning confocal microscope was used to obtain optical sections of 1–1.2- μ m thickness, which were used to construct maximum projection images of each brain.

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Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain

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In *Drosophila*, a 'clock' situated in the brain controls circadian rhythms of locomotor activity. This clock relies on several groups of neurons that express the Period (PER) protein, including the ventral lateral neurons (LN_vs), which express the Pigment-dispersing factor (PDF) neuropeptide, and the PDF-negative dorsal lateral neurons (LN_ds)¹. In normal cycles of day and night, adult flies exhibit morning and evening peaks of activity^{1,2}; however, the contribution of the different clock neurons to the rest-activity pattern remains unknown. Here, we have used targeted expression of PER to restore the clock function of specific subsets of lateral neurons in arrhythmic *per*⁰ mutant flies. We show that PER expression restricted to the LN_vs only restores the morning activity, whereas expression of PER in both the LN_vs and LN_ds also restores the evening activity. This provides the first neuronal bases for 'morning' and 'evening' oscillators in the *Drosophila* brain. Furthermore, we show that the LN_vs alone can generate 24 h activity rhythms in constant darkness, indicating that the morning oscillator is sufficient to drive the circadian system.

Many animals exhibit a bimodal activity distribution in the 24 h day/night cycle that is imposed by the rotation of the Earth³. Two circadian oscillators have been proposed to explain bimodal activity rhythms, and this theory has gained support from the finding of split free-running rhythms that have been reported in various organisms^{2,4–6}. It was shown recently that different regions of the mammalian suprachiasmatic nucleus (SCN) control morning and evening electrophysiological oscillations *in vitro*⁷, but no specific neuronal groups dedicated to morning or evening behaviours have been reported so far.

The morning and evening activity bouts displayed by *Drosophila* in 12/12 h light/dark (LD) conditions are controlled by the circadian clock, as inferred from the loss of anticipatory activity of both light transitions (lights-on morning transition and lights-off evening transition) in *per*⁰ mutants¹ (Fig. 1). Rhythmic behaviour is controlled by the brain, in which six groups of PER-expressing neurons have been described in the adult, including three subsets of lateral neurons (LN_s) and three subsets of dorsal neurons (DN_s)^{1,8} (Fig. 2a). In LD conditions, PER levels cycle similarly in these neuronal groups, showing a peak in the early morning and a trough at the end of the day^{9,10}. The two subsets of LN_vs (except one of the five small LN_vs (s-LN_vs)) express the PDF neuropeptide¹¹ (Fig. 2a), and their contribution to the rhythmic behaviour has been studied in *pdf*⁰ mutants¹² and LN_v-ablated flies^{12,13}. Flies devoid of functional LN_vs do not display lights-on anticipatory activity but show a robust phase-advanced evening peak^{12,13}, as shown here for *pdf*⁰ mutants (Fig. 1). These data indicate that PDF-expressing LN_vs are required for the morning activity but are dispensable for the evening activity. In constant darkness (DD), both *pdf*⁰ and LN_v-ablated flies become largely arrhythmic (although a variable proportion of the flies display a weak, short period rhythmicity), indicating that PDF-expressing LN_vs are required for robust 24 h free-running rhythms^{12,13}. To understand the contribution of the different neuronal groups to rhythmic behaviour, we undertook to express PER

specifically in clock neuron subsets of *per*⁰ flies and assay activity rhythms in LD and DD conditions. It has been shown that moderate levels of constitutive expression of *per* in *per*⁰ flies can restore low-amplitude PER protein cycling in the eye¹⁴. More importantly, robust rhythmic behaviour could be restored in *per*⁰ flies with pan-neuronal *elav-Gal4*-driven *per* expression¹⁵. We first verified that constitutive *per* expression in the brain clock neurons could restore a near-wild-type behaviour. A *cryptochrome* (*cry*)-*Gal4* line, which we have previously described as showing *Gal4*-driven *UAS-gfp* expression in the six clock neuronal groups of the adult brain¹⁶, was used to drive a *UAS-per* transgene in *per*⁰ flies. Indeed, *UAS-per* expression in *cry*-expressing neurons restores a complete LD behaviour to *per*⁰ flies, showing both morning and evening activity bouts (Fig. 1). We then tested the contribution of the PDF-expressing LN_vs by driving *per* expression using a *pdf-Gal4* transgene. *Pdf-Gal4*-driven *UAS-per* transcription restores PER protein expression specifically in the LN_vs of *per*⁰ flies, and reveals cycling of PER levels similar to the wild type, with high levels at Zeitgeber time (ZT) 0 and no staining at ZT12 (Fig. 2b; see also Supplementary Table S1). Flies with LN_v-restricted PER exhibited a clear morning activity that anticipated the lights-on transition, but no anticipatory evening activity (Fig. 1). We then used the *Mz520-Gal4* line (see Methods), whose expression in the adult brain is also restricted to the two subsets of PDF-expressing LN_vs (Fig. 2c; see also Supplementary Table S1). *Mz520-Gal4*-driven *per* expression in a *per*⁰ background shows PER cycling in the LN_vs, with a ZT0 peak and a ZT12 trough. As expected from the PER expression results, these flies exhibited the same LD profile as flies with *pdf-Gal4*-driven *per* expression, characterized by the loss of the lights-off anticipation (Fig. 1). Therefore PER expression in the PDF-expressing LN_vs is sufficient to restore the morning activity peak but not the evening activity peak.

We then used two *Gal4* lines expressed in various neuronal groups of the adult brain, including subsets of PER-expressing neurons. In both cases, *Gal4*-driven *UAS-per* expression in a *per*⁰ background resulted in the accumulation of PER protein only in the clock-cell-restricted fraction of the *Gal4* expression pattern (Fig. 2c, d; see also Supplementary Table S1). This is in agreement with the expression patterns of *per-Gal4* transgenes being much broader than the protein expression pattern⁸. It indicates that PER protein is unable to accumulate, at least to detectable levels, in neurons that do not express it in the wild type. No restoration of morning or evening anticipatory activity was observed in flies expressing *per* under the control of the *C929-Gal4* insertion (Fig. 1). *C929-Gal4* expression includes the large LN_vs (l-LN_vs) as the only clock cells¹⁷, and results in accumulation of PER only in the l-LN_vs in a *per*⁰ background (Fig. 2b; see also Supplementary Table S1). This result indicates that the large PDF neurons do not contribute to the anticipation of light transitions or require the small ones to do so. The *Mai179-Gal4* transgene is expressed in several groups of neurosecretory cells of the larval brain, including the PDF neurons¹⁸. In the adult brain, *Mai179-Gal4* is expressed in the five s-LN_vs (comprising four PDF-positive and one PDF-negative cell), a small number of l-LN_vs and three-four of the six PDF-negative LN_ds (Fig. 2d; see also Supplementary Table S1). As for the previous *Gal4* lines, expression in the dorsal neurons was never observed. Driving a *UAS-per* transgene with *Mai179-Gal4* in a *per*⁰ background results in PER accumulation in the s-LN_vs (occasionally in one l-LN_v) and LN_ds, with high levels at ZT0 and no labelling at ZT12 (Fig. 2d; see also Supplementary Table S1). The comparison of *Mai179-Gal4* with *pdf-Gal4* and *Mz520-Gal4* lines allows us to address the contribution of the PDF-negative LN_ds (namely those expressing *Mai179-Gal4*) to the LD behaviour. A very different behaviour was observed in the *Mai179-Gal4* (or *pdf-Gal4/Mai179-Gal4*) flies, which displayed a robust anticipation of both lights-on and lights-off transitions (Fig. 1). PER expression in the LN_vs and LN_ds therefore restores morning and evening activity bouts in

per⁰ flies. This result shows that PDF-negative LN_ds can induce evening behaviour, at least in the presence of PDF-expressing s-LN_vs. Although our results do not exclude contributions from other neurons in the wild type, we conclude that functional LN_vs are sufficient to generate morning activity, whereas adding LN_d function induces a second bout of activity at the end of the day. Because no obvious difference in PER cycling was observed between these neuronal groups, morning and evening activity peaks are probably controlled through the outputs of the lateral neurons. Similar conclusions have been reached using a different approach based on cell-specific ablation¹⁹.

The long period rhythms displayed by flies carrying a promoter-less *per* transgene with expression restricted to LN_vs and LN_ds suggests that lateral neurons are sufficient to drive some behavioural rhythmicity in constant darkness⁹. To understand the contribution of the LN_v and LN_d oscillators to sustained rhythmicity in the absence of light entrainment, we analysed PER oscillations and

locomotor behaviour of flies expressing PER in different lateral neuron subsets in DD conditions. As with wild-type flies, *per⁰* flies expressing *per* under the control of *pdf-Gal4* or *Mz520-Gal4* displayed sustained oscillations of PER protein levels in the s-LN_vs for up to 5 days in DD conditions, with the same decrease in amplitude (Fig. 3). In the three genotypes, PER levels rapidly decreased in the l-LN_vs (and was actually undetectable in the *Mz520-Gal4* line), although robust cycling was observed in these cells in LD conditions (see Fig. 2; see also Supplementary Table S1). This finding is in agreement with the reported loss of Timeless protein oscillations in the l-LN_vs of wild-type flies in DD conditions¹⁵. Not surprisingly, PER levels failed to cycle and stayed low in the l-LN_vs of the *C929-Gal4* flies. In the absence of any other PER-expressing neurons, the eight PDF-expressing s-LN_vs of the brain therefore behave as an autonomous oscillator in DD conditions. Very robust 24 h rhythms in DD conditions were found in flies expressing PER in the LN_vs only (Table 1). Indeed, the total

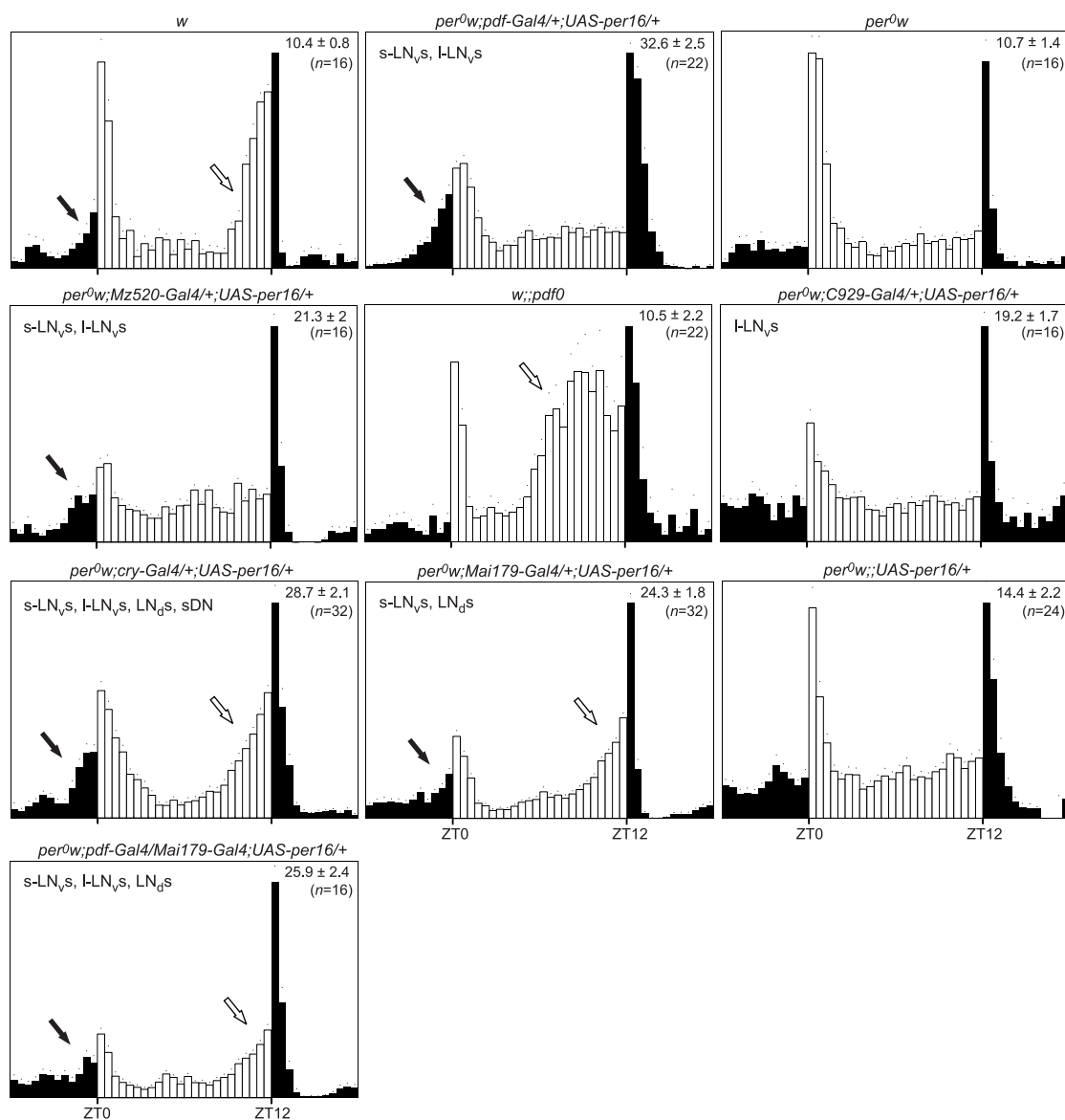


Figure 1 Locomotor activity in light/dark cycles. Histograms represent the distribution of activity through 24 h, averaged for *n* flies over three LD days. PER-expressing neurons (see Fig. 2) are indicated for each transgenic line. Open and filled bars indicate day and night phases, respectively. Activity levels are normalized independently for each genotype, and the mean daily activity is given in the upper right corner. Dots indicate the

s.e.m. of the activity for each 0.5 h. ZT is Zeitgeber time (12/12 h light/dark cycle; ZT0 is lights-on and ZT12 is lights-off). Filled and open arrows point to lights-on and lights-off anticipations, respectively. Note that genotypes devoid of anticipatory behaviour still respond to light by exhibiting high activity after the light transitions.

absence of PER staining in l-LN_s in the *Mz520-Gal4* line suggests that PER expression in the eight s-LN_s alone is sufficient to drive sustained 24 h rhythms in the absence of light cues. As expected from the loss of PER cycling in DD conditions, *C929-Gal4*-driven *per* expression in the l-LN_s could not restore any rhythmicity to *per⁰* flies. Surprisingly, *per* expression driven by *Mai179-Gal4* in the s-LN_s did not support PER protein accumulation in DD conditions, whereas robust oscillations persisted in the LN_ds (Fig. 3). This LN_d-restricted PER expression pattern could not

restore rhythmicity to *per⁰* flies (Table 1), supporting the requirement for PER-expressing LN_s to drive DD rhythmicity. Our results therefore indicate that the morning oscillator of the LN_s, but not the evening oscillator of the LN_ds, is able to control sustained behavioural rhythms in the absence of light/dark cycles.

We then asked how these two oscillators contribute to the building of the unimodal activity distribution in DD conditions, by looking at the phase of the activity bout (Fig. 4). Wild-type flies display a broad peak of activity centred in the second half of

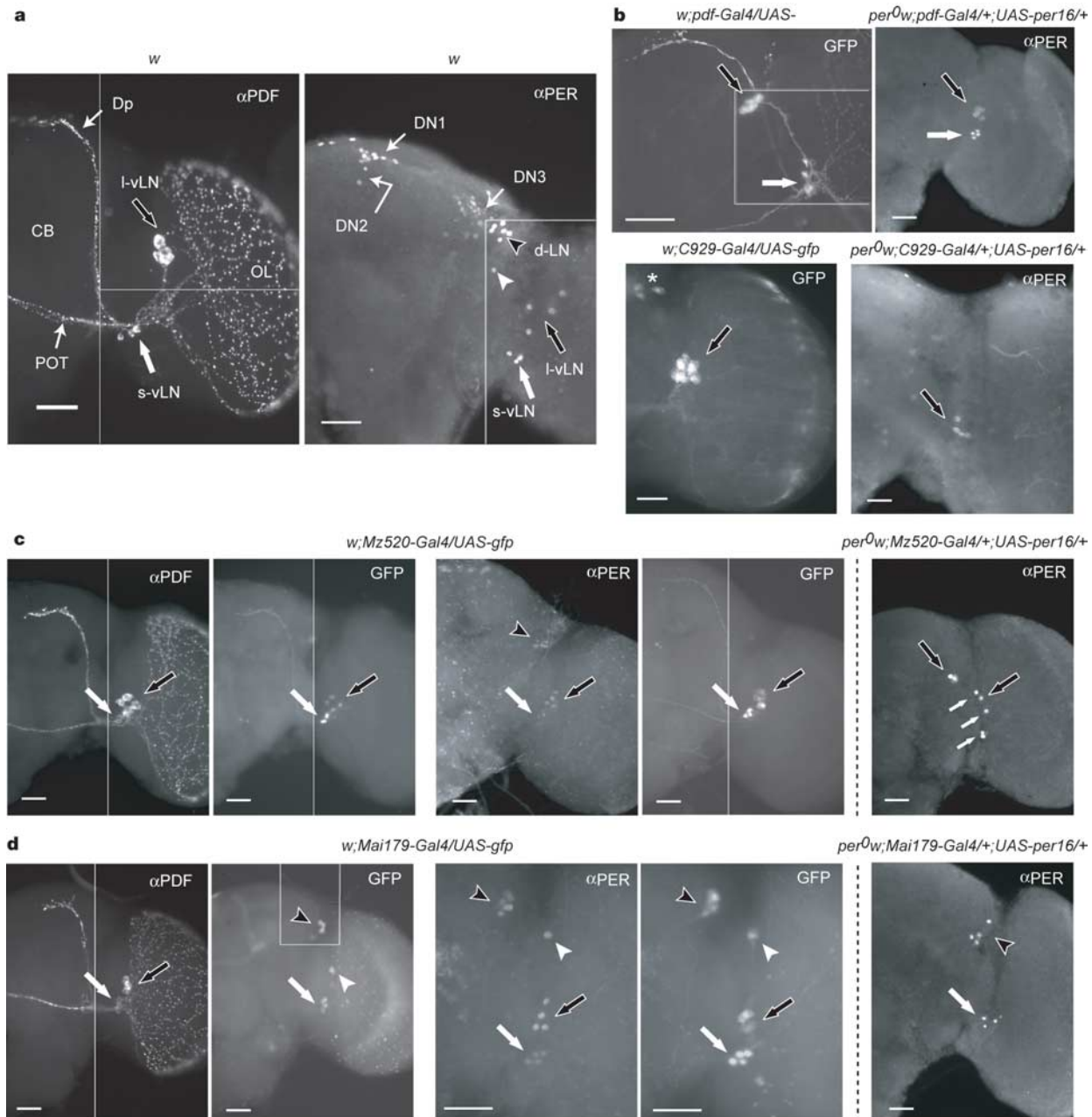


Figure 2 PER immunoreactivity of lateral neurons in light/dark cycles. All brain hemispheres are oriented with central brain (CB) on the left and optic lobe (OL) on the right. PDF-expressing s-LN_s, white arrows; l-LN_s, black arrows; single PDF-negative s-LN_s, white arrowhead; LN_ds, black arrowhead. Anti-PER immunolabellings were done at ZT0 (see Supplementary Table S1). **a**, Anti-PER (αPER) recognizes six groups of clock neurons in the wild-type adult brain (5 s-LN_s, ~4 l-LN_s, ~6 LN_ds, ~15 DN1 cells, 2 DN2 cells and ~40 DN3 cells)¹. Anti-PDF (αPDF) labels 4 s-LN_s and 4 l-LN_s. PDF-expressing s-LN_s send a dorsal projection (Dp), whereas l-LN_s send a projection through the posterior optic tract (POT) and arborize in the optic lobe¹¹. **b**, Top:

pdf-Gal4-driven GFP reporter in s-LN_s and l-LN_s¹². Anti-PER in *per*-rescued flies. Bottom: *C929-Gal4*-driven GFP in l-LN_s¹⁷. Asterisk indicates PER-negative cells expressing the transgene. Anti-PER in *per*-rescued flies. **c**, Left: anti-PDF and *Mz520-Gal4*-driven GFP co-expression. Middle: anti-PER and *Mz520-Gal4*-driven GFP co-expression. Right: Anti-PER in *per*-rescued flies. **d**, Left: anti-PDF and *Mai179-Gal4*-driven GFP co-expression. Middle: anti-PER and *Mai179-Gal4*-driven GFP co-expression. Right: anti-PER in *per*-rescued flies. Scale bars, 50 μm. White squares represent different focal plans, with anterior lateral neuron cell bodies and more posterior LN_v projections and dorsal neurons.

Table 1 Locomotor activity rhythms in constant darkness

Genotype	Total flies (n)	Rhythmic flies (%)	Period (h)	Power	Activity
w	16	16 (100)	24.0 ± 0.1	164 ± 7	24.5 ± 2.9
per ⁰ w	24	2 (8)	32.0 ± 0.0	30 ± 0	20.1 ± 2.0
w;;pdf0	16	4 (25)	24.0 ± 3.0*	37 ± 5	4.9 ± 1.2
per ⁰ w;;UAS-per16/+	16	2 (12)	20.2 ± 6.2	47 ± 4	18.3 ± 2.2
per ⁰ w;;cry-Gal4/+;UAS-per16/+	25	25 (100)	24.3 ± 0.2	91 ± 6	35.5 ± 4.2
per ⁰ w;;pdf-Gal4/+;UAS-per16/+	32	31 (97)	24.1 ± 0.3	113 ± 5	40.7 ± 4.8
per ⁰ w;;Mz520-Gal4/+;UAS-per16/+	32	32 (100)	24.2 ± 0.1	110 ± 7	18.0 ± 1.5
per ⁰ w;;C929-Gal4/+;UAS-per16/+	22	5 (23)	33.9 ± 4.8*	32 ± 3	18.0 ± 3.3
per ⁰ w;;Mai179-Gal4/+;UAS-per16/+	38	16 (42)	27.8 ± 2.0*	32 ± 2	13.2 ± 3.0
per ⁰ w;;pdf-Gal4/Mai179-Gal4;UAS-per16/+	32	32 (100)	24.2 ± 0.1	139 ± 7	32.7 ± 3.9
per ⁰ w;;Mz520-Gal4/Mai179-Gal4;UAS-per16/+	16	16 (100)	23.6 ± 0.1	141 ± 7	17.4 ± 1.0

The mean values of circadian period (h), associated powers (see Methods) and activities (number of events per 0.5 h) are given ± s.e.m.
*The highly variable periods of these genotypes indicate that they lack circadian rhythmicity, although a fraction of the individuals are found to be rhythmic because their power is weak but above threshold. Rhythmic individuals with aberrant periods are frequently observed in the per⁰ genotype as well.

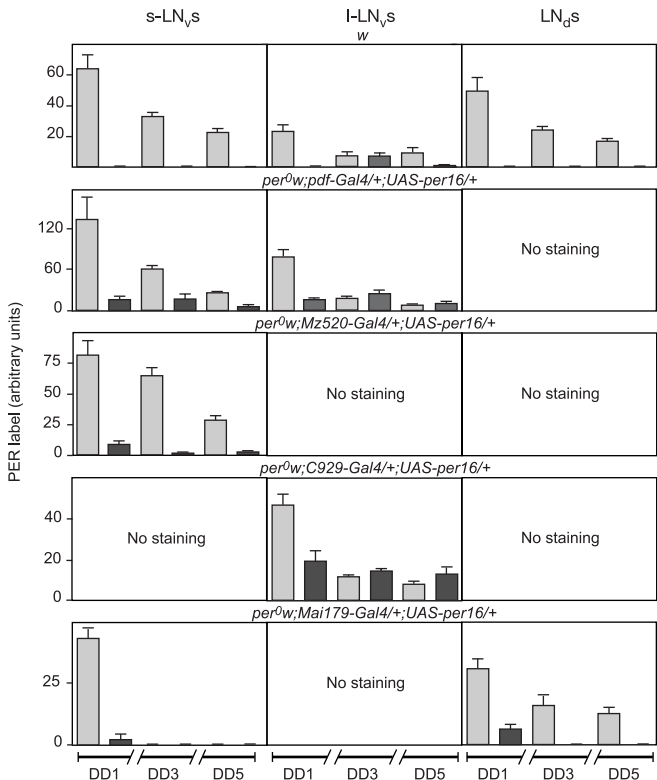


Figure 3 PER immunoreactivity of lateral neurons in constant darkness. Flies were entrained for 4 days in LD conditions (20 °C) and transferred to DD (circadian time (CT) 0 is 12 h after lights-off). Brains were dissected at CT 0 and 12 (DD1), CT 48 and 60 (DD3), and CT 96 and 108 (DD5). CT 0, 48 and 96 are in light grey, whereas CT 12, 60 and 108 are in dark grey. The quantification of PER labelling is reported in arbitrary units with the associated s.e.m. for each LN subset. Ten to fifteen brain hemispheres were analysed for each time point.

the subjective day, whereas flies with only a morning oscillator (*pdf-Gal4* or *Mz520-Gal4* alone) show an activity bout that is advanced by about ~6 h. Adding PER-expressing LN_ds (flies carrying *Mai179-Gal4* in addition to *pdf-Gal4* or *Mz520-Gal4*) extends the activity peak towards the end of the subjective day, similarly to flies expressing PER in all clock neuronal groups (*cry-Gal4*). Yet, the activity profile of these transgenic flies is different from the wild-type profile. We believe that the difference between wild-type flies and *per*⁰ flies expressing constitutive *per* in clock neurons may be due to relative levels of PER in the neurons that control morning and evening oscillators. This may also include contributing neurons from the dorsal neuron subsets and/or the single PDF-negative LN_v. Alternatively, the absence of circadian control of *per* transcription in

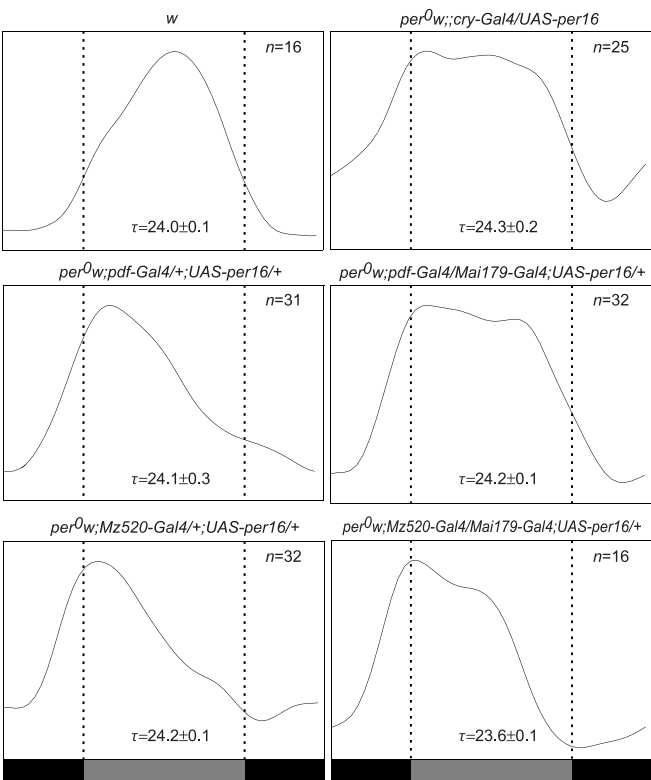


Figure 4 Locomotor activity in constant darkness. Waveforms represent the averaged distribution of activity through a circadian period (τ) window, for n rhythmic flies (see Table 1). τ windows are normalized to 24 h. Grey and black bars represent subjective day and night, respectively. Activity levels are normalized independently for each genotype (real activity values are given in Table 1).

the transgenic flies may influence the phase of output of the clock neurons and their interaction in constant darkness.

Our study shows that different clock neurons control morning and evening activity bouts in day/night conditions. In wild-type flies, the progressive loss of the morning peak when flies are transferred from LD to DD conditions, and the location of the activity peak in DD at the end of subjective day, suggested that the evening activity component in LD conditions was the major clock-regulated output². A different view emerges from these experiments, indicating that the morning oscillator is the autonomous pacemaker. However, changes in temperature or day length, as well as mutations of the core clock genes that change the circadian period, mostly affect the position of the evening peak¹. This suggests that complex interactions between the different neuronal groups that constitute morning and evening oscillators are required to respond

to environmental changes. Similarly to the control of diurnal and nocturnal activity by the mammalian SCN, understanding these interactions will require the characterization of the outputs of the clock neuron network. □

Methods

Fly strains

The *UAS-per16* insertion has been described previously¹³. *pdf-Gal4* is specifically expressed in both s-LN_s and l-LN_s¹², whereas *crs-Gal4-39* is expressed in the six clock neuronal groups of the adult brain: all LN_ss and LN_ds, most of the DN1 cells, the two DN2 cells and a fraction of the DN3 cells, in addition to several other neuronal groups that do not express clock genes¹⁶. Expression of the *C929-Gal4* enhancer trap has been described in about 100 peptidergic neurons of the adult brain, including the l-LN_ss, but not the s-LN_s¹⁷. *Mai179-Gal4* enhancer trap expression has been reported in the PDF neurons of the larval brain¹⁸, which become the s-LN_s of the adult brain (see ref. 8). The *Mz520-Gal4* enhancer trap²⁰ is expressed in the PDF neurons of the adult brain (H. Otsuna and K. Ito, personal communication). See Fig. 2 for the characterization of *Mai179-Gal4* and *Mz520-Gal4* expression in the adult brain.

Behavioural analysis

Experiments were carried out with 1–7-day-old males at 20 °C in *Drosophila* activity monitors (TriKinetics) as previously described^{13,21}. For DD analysis, flies were first entrained in 12/12 h LD cycles during 2–4 days, data were analysed for 6 days from the second day in DD. Data analysis was done with the Faas software 0.7.1, which is derived from the Brandeis Rhythm Package, and is available on request. Rhythmic flies were defined by χ^2 periodogram analysis with the following criteria (filter on): power ≥ 20 , width ≥ 2 h, with no selection on period value. Power and width are the height and width of the periodogram peak, respectively, and give the significance of the calculated period. Mean daily activity (number of events per 0.5 h) is calculated over the whole period in LD or DD conditions. Waveforms: individual fly waveforms were calculated from the 6 days of analysis and normalized to 24 h; group waveforms were then calculated by averaging those individual waveforms. All behavioural experiments were reproduced two or three times with very similar results.

Immunolabellings

All experiments were done on whole-mounted adult brains (only males for PER; unsorted males and females for PDF and GFP labellings). GFP reporter expression, anti-PER and anti-PDF labellings were done as previously described^{13,16,22}. Fluorescence signals were analysed with a Zeiss Axioplan2 epifluorescence microscope equipped with a SPOT2 (Diagnostic instruments) digital camera. Fluorescence intensity was quantified from digital images with the Adobe photoshop software. We applied the formula: $I = 100(S - B)/B$, which gives the fluorescence percentage above background (where S is fluorescence intensity and B is average intensity of the region adjacent to the positive cell).

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Role of histone H2A ubiquitination in Polycomb silencing

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Covalent modification of histones is important in regulating chromatin dynamics and transcription^{1,2}. One example of such modification is ubiquitination, which mainly occurs on histones H2A and H2B³. Although recent studies have uncovered the enzymes involved in histone H2B ubiquitination^{4–6} and a 'cross-talk' between H2B ubiquitination and histone methylation^{7,8}, the responsible enzymes and the functions of H2A ubiquitination are unknown. Here we report the purification and functional characterization of an E3 ubiquitin ligase complex that is specific for histone H2A. The complex, termed hPRC1L (human Polycomb repressive complex 1-like), is composed of several Polycomb-group proteins including Ring1, Ring2, Bmi1 and HPH2. hPRC1L monoubiquitinates nucleosomal histone H2A at lysine 119. Reducing the expression of Ring2 results in a dramatic decrease in the level of ubiquitinated H2A in HeLa cells. Chromatin immunoprecipitation analysis demonstrated colocalization of dRing with ubiquitinated H2A at the PRE and

promoter regions of the *Drosophila Ubx* gene in wing imaginal discs. Removal of dRing in SL2 tissue culture cells by RNA interference resulted in loss of H2A ubiquitination concomitant with derepression of *Ubx*. Thus, our studies identify the H2A ubiquitin ligase, and link H2A ubiquitination to Polycomb silencing.

In order to identify the E3 ligase responsible for H2A ubiquitination, we developed an assay that allows us to monitor the enzyme activity. In the presence of E1, E2, ATP and Flag-ubiquitin (F-Ub), HeLa nuclear protein fractions were tested for E3 ligase activity with core histone octamer or nucleosomal histone substrates. Western blot analysis of the reactions using an antibody against Flag revealed a positive signal around 25 kDa, the size of H2A plus F-Ub, indicating that a potential H2A E3 ligase activity is present in the corresponding fractions (lanes 19–21 of the rectangular area in Fig. 1a). The activity is nucleosomal histone specific as parallel experiments using core histone octamers failed to detect such an activity (Fig. 1a, lanes 8–10). A weak activity potentially for H2B was also detected (Fig. 1a, lane 20). Since the 0.5 M P11 fraction derived from nuclear pellet has the strongest activity, we thus focused our

further analysis on this fraction.

To verify that we have a real E3 ligase activity in the 0.5 M P11 fraction, we tested the dependence of the activity on each of the components in the reaction. Results shown in Fig. 1b indicate that the appearance of the ubiquitinated protein bands around 25 kDa depends on each component. To ascertain that the 25 kDa protein band is ubiquitinated H2A (uH2A), the protein band was subjected to mass spectrometry analysis which identified the protein as uH2A (Fig. 1c). Importantly, a peptide corresponding to the carboxy-terminal ubiquitin attached to Lys 119 of H2A was also identified. In addition, an antibody against uH2A recognizes the protein band specifically (data not shown). Thus, we conclude that we have identified an E3 ligase activity that specifically ubiquitinates H2A at Lys 119, a known *in vivo* ubiquitination site⁹.

We monitored the enzymatic activity through seven chromatography columns (Fig. 2a) in order to identify the protein(s) responsible for the H2A E3 ligase activity. After purification of the 0.5 M P11 fraction through DEAE5PW and Phenyl Sepharose columns, we determined the relative mass of the enzyme complex on the S300 gel-filtration column and found it to be about 250–

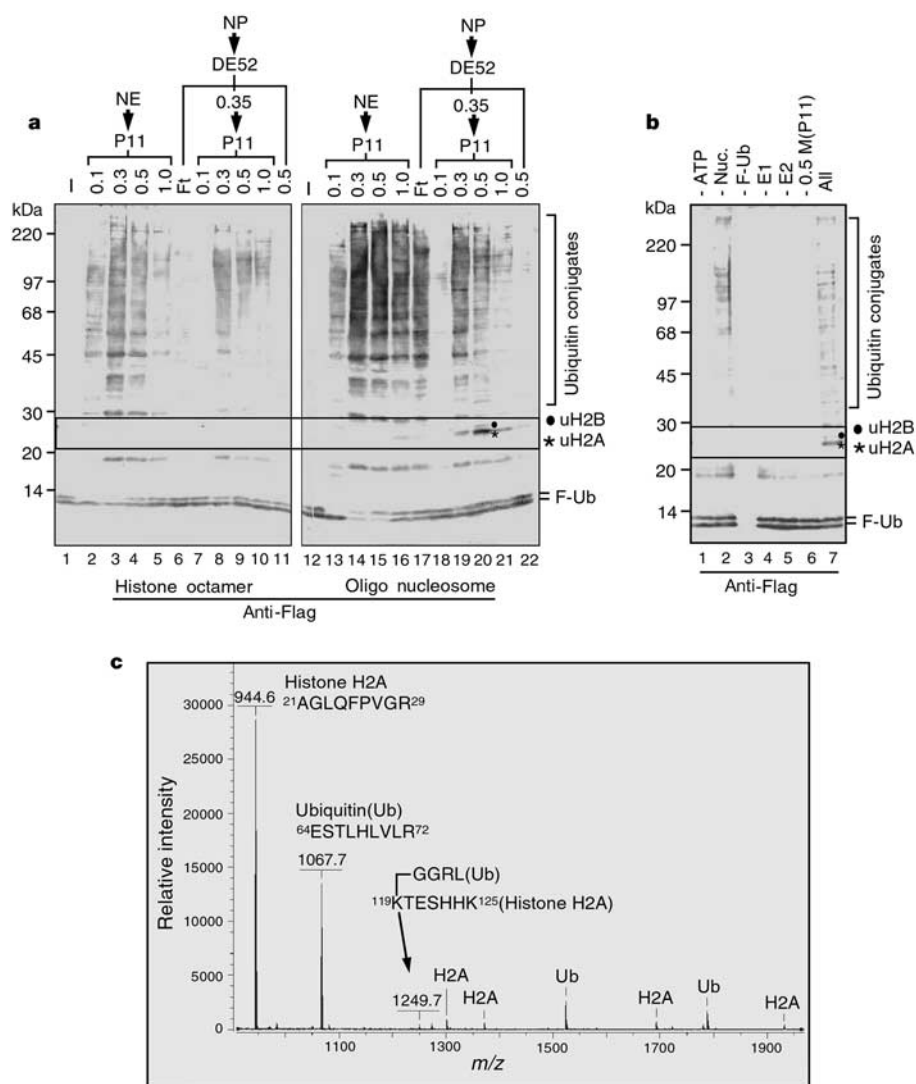


Figure 1 Identification of an H2A ubiquitin ligase activity in HeLa cells. **a**, Ubiquitin ligase assay using HeLa nuclear proteins fractionated on DE52 and P11 columns. Numbers on top of the panels indicate the salt concentration (M) for step elution. NE and NP represent nuclear extracts and nuclear pellet, respectively. Left and right panels use histone octamer and oligonucleosome substrates, respectively. **b**, The ligase activity depends on

the presence of ATP, E1, E2, ubiquitin, nucleosomal histones, and proteins present in the 0.5 M P11 nuclear pellet fraction. **c**, Mass spectrometry analysis of the ubiquitinated protein revealed the presence of histone H2A and ubiquitin. A peptide that contains amino acids from both ubiquitin and H2A identifies Lys 119 as the ubiquitination site.

300 kDa (Fig. 2b, top panel). Further purification on two additional columns allowed us to correlate four candidate protein bands (marked by * in Fig. 2c, top panel) with the enzymatic activity (Fig. 2c, second panel). The limited amount of samples prevented further purification of the complex. Thus, we pooled the MonoQ samples between fractions 29–32. After concentration, the samples were resolved using SDS–polyacrylamide gel electrophoresis

(SDS–PAGE) and the four candidate protein bands were recovered for identification. Mass spectrometry analysis identified the middle two protein bands as Ring1 and Ring2^{10,11}, two human homologues of *Drosophila* PRC1 core component dRing/Sce (Sex combs extra)^{12–14}. Western blot analysis confirmed that both proteins co-fractionate with the ligase activity on the MonoQ column (Fig. 2c, bottom two panels), as well as on the S300 column

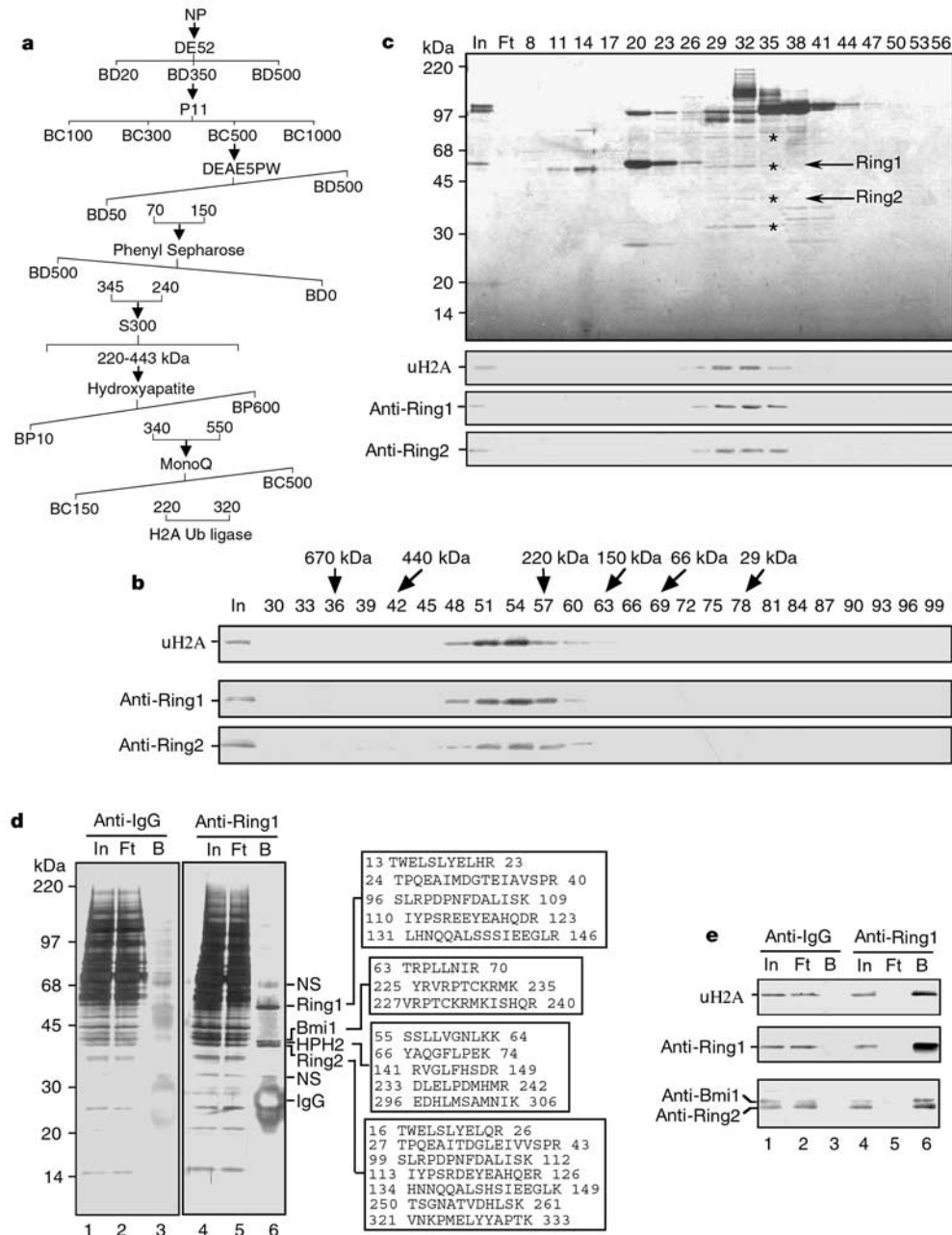


Figure 2 Purification and identification of the H2A ubiquitination ligase complex. **a**, Schematic representation of the steps used to purify the H2A ubiquitination ligase complex. Numbers represent the salt concentrations (mM) at which the E3 ligase activity elutes from the columns. **b**, The H2A ubiquitin ligase activity assay (top panel) and western blot analysis (bottom two panels) of the protein fractions derived from the gel-filtration S300 column. The elution profile of the protein markers is indicated on top of the panel. Antibodies used for the western blot are indicated. **c**, Silver staining of a polyacrylamide–SDS gel (top panel), H2A ubiquitin ligase activity assay (second panel) and western blot analysis (bottom two panels) of the fractions derived from the MonoQ column. The candidate proteins that co-fractionated with the E3 ligase activity are indicated by *. The

positions of the protein size markers on SDS–PAGE are indicated to the left of the panel. **d**, Silver staining of polyacrylamide–SDS gels containing samples derived from input (In), flow-through (Ft), and bound (B) using purified rabbit IgG and Ring1 antibodies. The identities of the proteins co-immunoprecipitate with Ring1 are indicated. Peptides derived from each protein band are also indicated. Numbers represent the amino acid number of the respective proteins. NS indicates nonspecific-binding proteins that are also present in the control IP (lane 3). **e**, The same samples presented in **d** were analysed by H2A ubiquitin ligase activity assay (top panel) and western blotting (bottom two panels). Antibodies used are indicated.

(Fig. 2b, bottom two panels). However, we failed to unequivocally identify the other two protein bands.

To define the composition of the E3 ligase complex, we performed immunoaffinity purification using an aliquot of the hydroxyapatite input material and an antibody against Ring1. The affinity-purified samples were split for silver staining, ubiquitin

ligase assay, western blotting, and protein identification. Silver staining revealed that anti-Ring1 antibodies specifically immunoprecipitated four protein bands (Fig. 2d, compare lanes 6 and 3). Mass spectrometry analysis identified these proteins as Ring1, Bmi1, HPH2 (human Polyhomeotic 2) and Ring2 (Fig. 2d). The identities of these proteins were also verified by western blotting (Fig. 2e, bottom two panels). The protein complex is apparently responsible for the ligase activity as the activity is depleted in the flow-through (Fig. 2e, top panel, compare lanes 5 and 2). Collectively, the above results allow us to conclude that the H2A ubiquitin E3 ligase complex is composed of four Polycomb group (PcG) proteins including Ring1, Bmi1, HPH2 and Ring2. Given that a human Polycomb repressive complex containing Ring1, Ring2, Bmi1, HPH1, HPH2, HPH3, M33, SNF2H, SCM1, HSP70 and tubulin has been previously purified from HeLa cell lines expressing Flag-

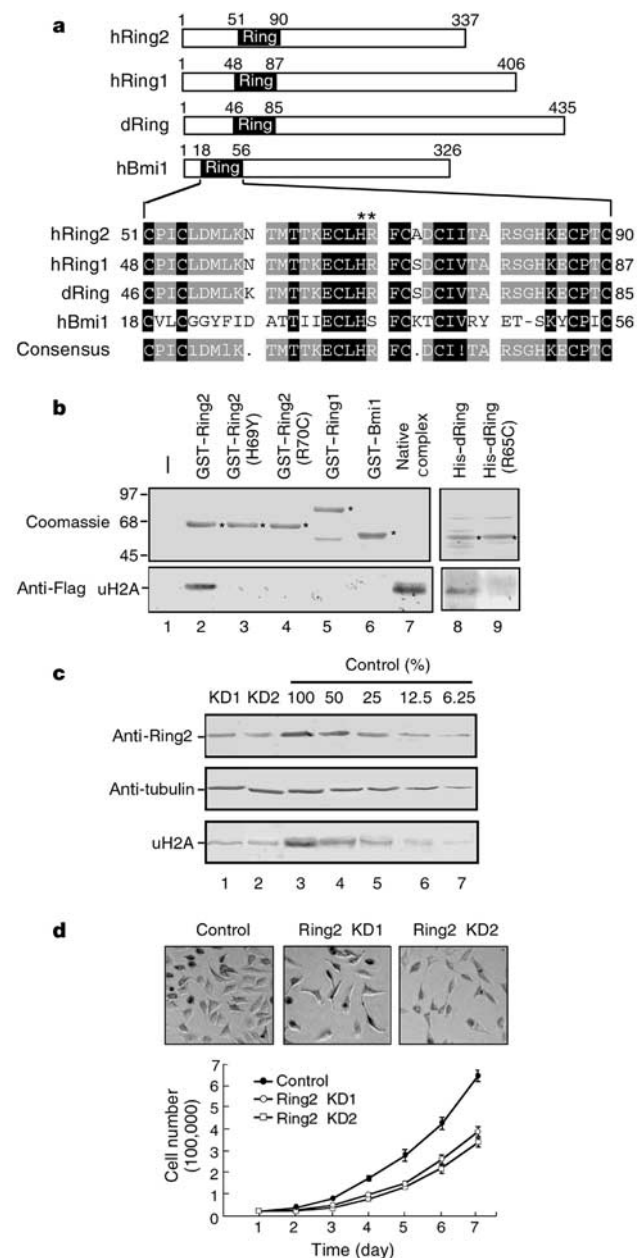


Figure 3 Ring2 is the catalytic subunit and is required for H2A ubiquitination *in vivo*.

a, Diagram of human Ring1 and Ring2, their *Drosophila* homologue dRing, and human Bim1. A sequence alignment of the RING finger domain of these proteins is shown. Amino acids that are completely conserved are shaded with black. The two Ring2 mutants used in **b** are indicated by *. Numbers represent the amino acid number. **b**, Ubiquitin ligase activity (bottom panel) of recombinant proteins (top panel, lanes 2–6, 8 and 9) and native hPRC1L (lane 7). Coomassie (middle panel) and Anti-Flag uH2A (bottom panel) staining are shown. **c**, Estimation of the Ring2 knock-down level in the KD1 and KD2 cell lines relative to the mock knock-down cells transfected with empty vector. Tubulin was used as a loading control. Antibodies used are indicated on the left of the panel. **d**, Ring2 knock-down results in morphological change and cell growth inhibition. Top panels show morphological changes of control and knock-down HeLa cells. Bottom panel shows the growth curve of control and knock-down HeLa cells. Viable cells were counted by trypan blue staining at different times after initial seeding of 3×10^4 cells.

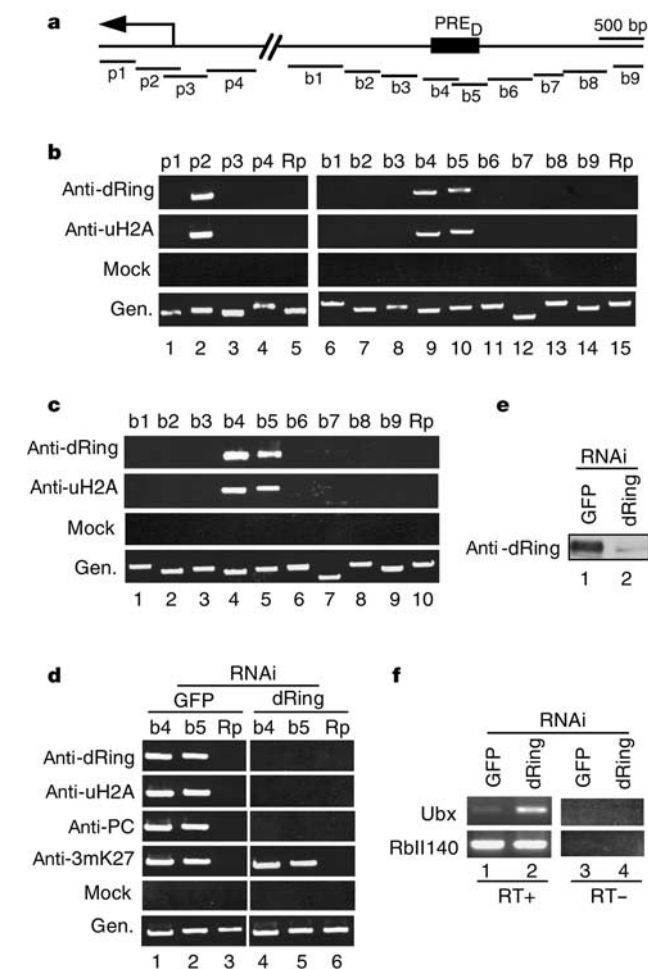


Figure 4 dRing-dependent H2A ubiquitination at *Ubx* PRE and promoter regions.

a, Schematic representation of the *Ubx* promoter and *bxd* PRE regions. The regions amplified by PCR in the ChIP assays are depicted as horizontal lines. **b**, ChIP results showing the wild-type distributions of dRing and ubiquitinated H2A in wing imaginal discs. **c**, ChIP results showing the distribution of dRing, and ubiquitinated H2A at the PRE region in normal SL2 cells. **d**, ChIP results of SL2 cells transfected with GFP dsRNA (left panels) or dRing dsRNA (right panels). **b–d**, Antibodies used in immunoprecipitations and the PCR amplified regions are indicated to the left and above, respectively. Rp, Rpl140 promoter; mock, crude rabbit preimmune-antisera; Gen, genomic DNA. **e**, Western blots showing dRing levels in SL2 RNAi cells. **f**, dRing knock-down leads to derepression of *Ubx*. Levels of *Ubx* transcripts in cells transfected with GFP or dRing dsRNA was evaluated by RT-PCR. **e** and **f**, SL2 cells transfected twice with GFP or dRing dsRNA. Rpl140 expression serves as a control. RT +, reverse transcriptase included in the reaction; RT -, reverse transcriptase omitted.

tagged Bmi1 or M33 and was named hPRC1¹⁵, we have named our complex hPRC1L (human PRC1-like) to reflect the similarity of these two protein complexes.

RING finger has been identified as a signature motif for ubiquitin E3 ligase¹⁶. Of the four PcG proteins in the H2A ubiquitin E3 ligase complex, three contain RING fingers (Fig. 3a). To determine the catalytic subunit of the E3 ligase complex, we expressed each of the ring-containing proteins as a GST fusion protein (Fig. 3b) and analysed their potential ubiquitin ligase activity using equal amounts of oligonucleosomal substrates. Results shown in Fig. 3b indicate that only recombinant Ring2 is active (bottom panel, compare lanes 2, 5 and 6). To determine whether the RING finger domain of Ring2 is responsible for the enzymatic activity, a conserved His residue involved in zinc binding was mutated to Tyr (H69Y). This change completely abrogated the E3 ligase activity (Fig. 3b, compare lanes 2 and 3), indicating that the RING finger domain of Ring2 is critical for its ubiquitin ligase activity. Previous studies in *Drosophila* have identified a loss of function *dRing/Sce* allele, *Sce*^{33M2}, that contains a single amino acid substitution (R65C) in the RING finger domain¹². To analyse the effect of this mutation on the E3 ligase activity, we generated a Ring2 mutant (R70C) that mimics the *Sce*^{33M2} mutation (Fig. 3a). Interestingly, this mutation also abrogated the Ring2 enzymatic activity (Fig. 3b, compare lanes 2 and 4), raising the possibility that the phenotypes associated with *Sce*^{33M2} may be linked to defective H2A ubiquitination. To verify this possibility, wild type and a mutant dRing (R65C) that has the same mutation as that of the *Sce*^{33M2} allele were made and tested for E3 activity. Results shown in Fig. 3b (lanes 8 and 9) demonstrate that wild-type dRing, but not the mutant, has H2A ubiquitin ligase activity. Therefore, we conclude that both Ring2 and dRing contain intrinsic E3 ligase activity and that the conserved Arg 70 (Arg 65 in dRing) in the RING domain is critical for the enzymatic activity.

To investigate the role of Ring2 in H2A ubiquitination *in vivo*, we generated two independent stable Ring2 knock-down cell lines, KD1 and KD2, that target two different regions of human Ring2 messenger RNA for degradation using a vector based short interfering RNA (siRNA) approach¹⁷. Western blot analysis indicated that about 75% knock-down was achieved at the protein level in both cell lines (Fig. 3c, top panel, compare lanes 1 and 2 with 5). To evaluate the effect of Ring2 knock-down on H2A ubiquitination *in vivo*, we performed western blot analysis using a previously well-characterized¹⁸ uH2A-specific monoclonal antibody E6C5, which can also recognize ubiquitinated H2A from *Drosophila* (Supplementary Fig. S1). Results shown in Fig. 3c indicated a roughly 75% decrease in the uH2A level in the knock-down cells when compared with that of control knock-down cells (bottom panel, compare lanes 1 and 2 with 5). These data strongly suggest that Ring2 is required for H2A ubiquitination *in vivo*. Similar to knock-down of SUZ12¹⁹, an EED-EZH2 HMTase component, Ring2 knock-down resulted in changes in cellular morphology and slower growth (Fig. 3d), consistent with a role of Ring2 in regulating cellular differentiation and proliferation²⁰.

To understand the functional relationship between dRing-mediated H2A ubiquitination and Hox gene silencing, we focused our study on a well-characterized *Drosophila* PcG target gene, *Ubx*. Silencing of *Ubx* transcription in wing imaginal discs requires both the ESC-E(Z) and PRC1 complexes, in addition to the major PRE (Polycomb response element) within the *bxd* regulatory region (PRE_D) to which the two complexes bind. Having demonstrated the specificity of the E6C5 monoclonal anti-uH2A antibody for *Drosophila* uH2A (Supplementary Fig. S1), we performed chromatin immunoprecipitation (ChIP) assays of wing imaginal disc cells which revealed colocalization of dRing and ubiquitinated H2A at PRE_D and immediately downstream of the *Ubx* transcription start site (Fig. 4b), sites at which E(Z) and PC, another PRC1

core component, have previously been shown to be located^{21,22}. Homozygous *Sce/dRing* mutants die early in development, precluding ChIP analysis of mutant wing imaginal discs¹². Therefore, dependence of H2A ubiquitination at PRE_D on dRing was tested using SL2 tissue culture cells.

Previous studies have shown that PC, E(Z) and E(Z)-dependent H3-K27 methylation colocalize at the PRE_D region in SL2 cells^{21,22}. As in wing imaginal discs, dRing and ubiquitinated H2A also colocalize at PRE_D (Fig. 4c). Depletion of dRing by RNAi resulted in loss of ubiquitinated H2A, as well as PC from the PRE (Fig. 4d, right panels). E(Z)-dependent trimethylation on H3-K27 was not affected (Fig. 4d, right panels). Loss of dRing, PC and uH2A is accompanied by derepression of *Ubx* gene expression (Fig. 4f), similar to that previously observed upon RNAi-mediated depletion of PC²². Because the PRC1 complex appears to be lost along with dRing, we cannot distinguish between the roles of H2A ubiquitination and other potential functions of the PRC1 complex in *Ubx* repression. However, somatic clones that are homozygous for the *Sce*^{33M2} allele, which contains the R65C substitution within the RING domain, exhibit *Ubx* derepression in wing imaginal discs¹². Together with the fact that the R65C mutation abolishes dRing ubiquitin ligase activity (Fig. 3b), these observations collectively suggest that dRing-mediated H2A ubiquitination plays an important role in *Ubx* gene silencing.

It is intriguing that ubiquitination of H2A and H2B has opposite effects on transcription. In the case of H2B, ubiquitination is associated with gene activation^{23,24}, and is required for H3-K4, -K79 methylation, a phenomenon referred to as 'trans-tail' regulation^{7,25,26}. It is yet to be determined whether H2A ubiquitination also participates in trans-tail regulation since H3-K27 methylation, a modification that is central to PcG transcriptional silencing, appears to be independent of H2A ubiquitination (Fig. 4d, compare left and right panels). It is possible that H2A ubiquitination may regulate other histone modifications that are associated with PcG-dependent silencing or inhibit modifications required for transcriptional activation. Although the mechanism by which H2A ubiquitination contributes to PcG silencing is currently unknown, identification of the responsible enzyme should allow us to better understand the PcG silencing system. □

Methods

In vitro histone ubiquitin ligase assay and ubiquitination site mapping

Histone octamers (5 µg) or oligonucleosomes (5 µg) were incubated with 20 µl protein fractions or recombinant proteins in a 36 µl reaction containing 50 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 2 mM NaF, 0.6 mM DTT, 2 mM ATP, 10 µM Okada acid, 0.1 µg ubiquitin activating enzyme E1 (Calbiochem), 0.6 µg ubiquitin conjugating enzyme Ubc5c, 1 µg flag-ubiquitin (Sigma). After incubation at 37 °C for 1 h, reaction was terminated by addition of SDS-PAGE loading buffer. The proteins were resolved in 8–15% SDS-PAGE and blotted with the anti-Flag antibody. For ubiquitination site determination, a protein band corresponding to ubiquitinated H2A was excised and subjected to trypsin digestion and MALDI-TOF MS analysis²⁷. Identification of a peptide that corresponds to residues 119–125 of H2A (K*TESHHK) linked to (indicated by *) the C-terminal LRGG of ubiquitin suggests that Lys 119 is the site of ubiquitination.

Constructs and antibodies

Plasmids encoding GST–Ring2 and GST–Ring2 (H69Y) were obtained from S. Kang¹⁰. The GST–Ring2 (R70C) mutant was generated by PCR-based mutagenesis and confirmed by DNA sequencing. Plasmids encoding GST–Ring1 and GST–Bmi1 were constructed by PCR amplification of EST clones (CS0DI076YE14 and CL0BB004ZD04) and the inserts cloned into *EcoRI* and *XhoI* sites of pGEX-KG vector. A plasmid encoding His₆-dRing was constructed by RT-PCR amplification of the RNA from *Drosophila* embryos and the full-length coding sequence inserted into the pQE30 vector. The His₆-dRing (R65C) mutant was generated by replacing a *PstI* fragment of wild-type dRing with a corresponding fragment from the mutant *Sce*^{33M2} allele. All the sequences were verified by DNA sequencing. Plasmid for His₆-Ubc5c was a gift from Y. Xiong. Antibodies against uH2A and Bmi1 were purchased from Upstate. Antibodies against PC, H3-3mK27, Ring1 and Ring2 have been previously described^{22,28,29}. Antibody against dRing was generated in rabbits by injection of a His₆-dRing fusion protein.

dRing knock-down and ChIP assays

Culture of SL2 cells and transfection with double-stranded RNA, ChIP assays and RT-PCR analysis of *Ubx* transcripts from SL2 cells were performed as previously described²².

with the exception that ChIP assays were performed on cells harvested 72 h following a single transfection with dsRNA. *dRing* dsRNA including exonic sequences extending from 167 to 1,154 base pairs (bp) downstream of the ATG was synthesized by bi-directional transcription of RT-PCR products containing T7 promoter sequences at both ends. Isolation of wing imaginal discs and ChIP assays were performed as previously described²².

Information for purification and identification of histone H2A ubiquitin ligase complex, for generation and characterization of Ring2 knock-down cell lines, as well as for the specificity of the uH2A antibody, is available in Supplementary Methods and Supplementary Data.

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corrigenda

The lipid phosphatase SHIP2 controls insulin sensitivity

S. Clément, U. Krause, F. Desmedt, J.-F. Tanti, J. Behrends, X. Pesesse, T. Sasaki, J. Penninger, M. Doherty, W. Malaisse, J. E. Dumont, Y. Le Marchand-Brustel, C. Erneux, L. Hue & S. Schurmans

Nature **409**, 92–96 (2001).

In this Letter, we investigated the production and the phenotypic characterization of a SHIP2 (SH2 domain containing inositol phosphate 5-phosphatase type 2, or *Inpp1l*) knockout mice. Total or partial loss of SHIP2 enzyme in these mice resulted in an increased insulin sensitivity. From these experiments, we concluded that SHIP2 is a potent negative regulator of insulin signalling and insulin sensitivity *in vivo*. However, we have recently realized that the 7.3-kilobase genomic DNA fragment deleted in these mice includes, in addition to exons 19–29 of the *SHIP2* gene, the third (and last) exon of the *Phox2a* gene. The deletion of this exon results in the absence of the 124 carboxy-terminal amino acids from a total of 280, including part of the homeodomain, and should give rise to a completely non-functional Phox2a protein if expressed. As a consequence, the mice we described have both *SHIP2* and *Phox2a* genes inactivated. It is currently unknown whether the increased insulin sensitivity we observed in our mice results from the inactivation of the *SHIP2* gene alone, of the *Phox2a* gene alone, or of both genes. □

Induction of DNA methylation and gene silencing by short interfering RNAs in human cells

Hiroaki Kawasaki & Kazunari Taira

Nature **431**, 211–217 (2004).

In the Methods section of this Letter, the published primer sequences used to amplify the E-cadherin and *erbB2* promoters for bisulphite sequencing were incorrect. We used two primer sets, one for unconverted DNAs and the other for converted DNAs. Primers for unconverted DNAs were: for the E-cadherin promoter, the forward primer was 5'-TCTAGAAAAATTTTAAAAA-3' and the reverse primer was 5'-CAGCGCCGAGAGGCTGCGGCT-3'; for the *erbB2* promoter, the forward primer was 5'-CCTGGAAGCCA-CAAGGTAAAC-3' and reverse primer was 5'-TTTCTCCGGTCCCAATGGAGG-3'. Primers for converted DNAs were: for the E-cadherin promoter, the forward primer was 5'-TTTA-GAAAAATTTTAAAAA-3' and the reverse primer was 5'-CAACACCAAAAACTACAAC-3'; for the *erbB2* promoter, the forward primer was 5'-TTTGGAAGTTATAAGGTAAAT-3' and the reverse primer was 5'-TTTCTCCAATCCCAATAAAAAA-3'. □

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Key words

Academic search committees, human-resource professionals and, increasingly, computer programs that monitor online job applications all scan resumés for key words to check that an applicant's skills match up with the advertised position's demands, panellists at a *Naturejobs*-sponsored career fair in Boston said last week. But they also emphasized that specific technical skills are not as important as 'soft' skills such as the ability to communicate, solve problems and work in a team. After all, once-esoteric technical skills such as cloning a gene are now passé.

Several of the 400 or so postdocs, graduate students and mid-career scientists attending the event thought that these two approaches were contradictory. The tone of the questions, as well as their repetition over two sessions, were tinged with undercurrents of fear — mixed with frustration — that not knowing some magic words would keep them from a position for which they were well qualified.

So how should they resolve this apparent inconsistency? By realizing that specific scientific and technical skills and soft skills are not mutually exclusive, several panellists said. Having both — and, more important, showing in resumés, interviews and follow-up conversations that you have them, and that they match the needs of the organization — is the most important thing.

Perhaps rather than thinking of hard and soft skills as opposites, applicants should think of demonstrating their mastery of both in a two-step process. Illustrating hard skills in a resumé can unlock the barrier between you and an interview. But showing how you used soft skills to solve a particular scientific problem or move along a project can help you walk through the door to a position.

Paul Smaglik
Naturejobs editor



Contents

CAREERS AND RECRUITMENT

The bottom line
for biostatistics p880

CAREER VIEW

Bricks & Mortar
Rensselaer Polytechnic Institute
Graduate Journal
The sound of science
Movers
David Lane p882

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Information on the
scientific job market

FOCUS

SPOTLIGHT

RECRUITMENT

ANNOUNCEMENTS

EVENTS

Analyse this

As key players on scientific teams, biostatisticians are in high demand. Kendall Powell sums up the situation.

In the 1950s, Edwards Deming, a statistician known as the father of the Japanese industrial revolution, almost single-handedly reformed the country's economy by introducing a mathematical concept: set quality standards so high that the likelihood of failure is near impossible. That concept now pervades the biosciences. In industry, it is essential for monitoring the safety of drugs and, in basic science, it is necessary for drawing causal relationships conclusively, for instance, between multiple genes and a single disease.

Although familiarity with such ideas is vital, people who can handle them — and understand the problems their colleagues are trying to solve — are in short supply. As a result, they hold powerful positions in academic research, and the pharmaceutical and biotech industries. Today, biostatistics offers lucrative salaries and a variety of projects that go beyond simple 'number crunching'. And yet few people in the scientific community are aware of it.

The boom, say those in the know, can be attributed to the exponential growth in data sets: as biology becomes more of a quantitative science, those with solid foundations in statistics and mathematics are getting the pick of positions.

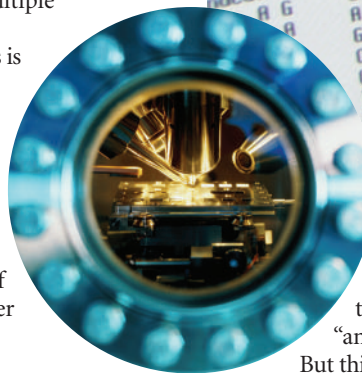
"Somebody with an interest in mathematics and science can be pretty sure to get a job," says Bradley Efron, a Stanford University statistician and president of the American Statistical Association (ASA). The combination of undergraduate work in biology or chemistry with a graduate degree in statistics is very attractive, agree industry statisticians.

Stats after a science degree

"Why?" is the question statistics-degree seekers frequently find themselves answering, especially when they have already secured one advanced degree in the sciences. When cosmologist Jim Crooks and his neuroscientist wife, Kristy, realized they "had a little nerdling on the way", Crooks started thinking seriously about job security, because academic cosmology positions are rare. Deciding he might like to work at the Centers for Disease Control and Prevention in Atlanta, Georgia, and banking on solid mathematics skills from his physics doctorate, Crooks has enrolled in the master's programme in statistics at the University of North Carolina in Chapel Hill.

For Stephanie Kane, working as a lab technician after finishing a master's degree in zoology revealed her true calling as a statistician. After being handed piles of data to analyse, Kane had to teach herself stats on the fly. Doing so, she realized that she liked figuring out the right way to pull information from the numbers. Now pursuing a master's in statistics at Washington State University in Pullman, Kane says her former biology colleagues ask her to clarify statistical questions in their own data.

K.P.



Demand for statisticians is flourishing as biology embraces the large data sets provided by devices such as time-of-flight mass spectrometers.

Efron notes that having solid training in both biology and statistics is "an ideal only approached by a few people".

But this may change as more science graduates turn to statistics programmes (see 'Stats after a science degree'), creating the next generation of analysts, who will tackle complex projects such as gene-environment interactions in cancer and personalized medicine.

SOCIAL SCIENTISTS

The image of a statistician toiling away in obscurity behind a computer after being handed a pile of data could not be farther from the truth. Almost every biostatistician plays a crucial role in a team of researchers — whether collaborating on an academic project or developing a clinical trial protocol. Introverts with limited people skills will find themselves struggling to do their job adequately. Biostatisticians have to be able to explain to others why they chose a particular analysis and how to interpret the analysed results.

"The more lingo you understand of the people you are working with, then the more efficiently you can operate," says Daniel Mowrey, a biostatistician with Elanco, the animal health division of Eli Lilly in Greenfield, Indiana. Mowrey, who has a BSc in maths and chemistry and a doctorate in statistics, says that communicating with field scientists lets him pinpoint variation in their studies and suggest changes to make experiments more precise.

"It is not at all 'crunching numbers,' because we have powerful computers to do that," says Fabio Macchiardi, director of biostatistics at the biotech company Serono Genetics Institute in Evry, France, and a professor at the universities of Toronto and Milan. Instead, he says, biostatistics is much more of an "innovative



exploration” to find out what information can be extracted about diseases or treatments. And the collaborative nature of the work means that statisticians experience many types of projects.

Katherine Monti, a 27-year industry veteran who directs the Boston office of the contract research organization Rho, reports in ASA’s *AmStat News* that her projects have ranged from optimizing Twinkie springiness to developing a breeding database for a colony of 11,400 cats and working on AIDS drug trials. “Sure, I have boring spells,” she says, “but some new project always comes along to save the day.”

Monti’s experiences reflect the array of opportunities for biostatisticians in industry. Positions designing and analysing clinical data make up the lion’s share of jobs. But biostatisticians can also be involved at all stages from discovery to the point at which the drug is submitted to regulatory agencies.

EXPERIMENTAL DESIGN ON A DIME

It is up to statisticians to give companies the most bang for their buck in experimental terms, by designing protocols that will generate the least amount of variation, Mowrey explains. When drugs are given to food-producing animals, any residues in meat or milk are meant to be proved safe for public consumption. So Mowrey ensures that the necessary safety studies are as comprehensive as possible.

Jerry Weaver, manager of statistics at Pfizer’s research and development facility in Groton, Connecticut, discusses statistical strategies with clinical researchers. He and Mowrey both note that statisticians play a huge role in the ‘go/no-go’ decisions that decide a pharmaceutical company’s direction. Although, Weaver



Mitchell Gail (left) and Fabio Macchiardi see biostatistics as an area in which innovation and exploration are key.

points out, statisticians do often need to take on significant management or administrative duties to progress further in a drug company.

Other industry options include working on quality control of medical devices or diagnostic tests, or optimizing foods and fertilizers, all of which require quantification of biological variation. In these areas, where industry standards are already clearly defined, a statistician can succeed with on-the-job biology training.

But another burgeoning sector of biostatistics — genetic epidemiology and bioinformatics — requires a bit more background in the biomedical sciences. “Newer types of statistics are called for with devices such as microarrays and time-of-flight mass spectrometers,” which can generate millions of data points per patient, says Efron.

CROSS TRAINING

For these situations, a biostatistician needs to understand both statistical theory and the use of bioinformatic tools, says Macchiardi. The bioinformation age will bring new challenges to cancer epidemiology, agrees Mitchell Gail, biostatistician in the Division of Cancer Epidemiology and Genetics at the National Cancer Institute in Rockville, Maryland. Gail helped create a statistical model to predict a

woman’s absolute risk of developing breast cancer during her lifetime. He says it will take new statistical methods to address how much of cancer is explained by specific genes.

Familiarity with handling scientific data and forming hypotheses “would be a distinct plus”, says Gail, for anyone considering work at academic or medical centres. Although double degrees in science and statistics are not required for

most of these jobs, an aptitude for mathematics is a must. But take heart, says Vicki Hertzberg, a graduate adviser in the biostatistics programme at Emory University’s Rollins School of Public Health in Atlanta, Georgia: a year of calculus and linear algebra and high quantitative test scores would earn a biologist strong consideration for a place.

Michael Kutner, who chairs the Emory programme, explains that, unlike straight statistics courses, a biostatistics degree focuses equally on theory and applied statistics. The programme places students in research collaborations on campus with real, messy data, he says, to give them practical experience.

The rewards for statistics graduates in the biological sciences are at an all-time high. Half the job posters on the ASA’s website are for biostatisticians, split almost evenly between industry and academic placements. According to the ASA’s 2003 salary survey, biostatistics researchers started on about \$75,000 and could make upwards of \$166,000 as full professors. In industry, a master’s level statistician might start at \$65,000 and a PhD with 20 years’ experience could pull in as much as \$175,000. Couple those numbers with the high job satisfaction of ever-changing projects, and brushing up on your maths suddenly doesn’t sound so bad. ■

Kendall Powell is a freelance science writer based in Broomfield, Colorado.

Web links

American Statistical Association

♦ www.amstat.org

Rollins School of Public Health’s
Department of Biostatistics

♦ www.sph.emory.edu/hpbios.html

National Cancer Institute’s
Division of Cancer Epidemiology
and Genetics

♦ dceg.cancer.gov/biostat.html

Serono Genetics Institute

♦ www.genetics.serono.com

GRADUATE JOURNAL

The sound of science

As an undergraduate, my first summer research was done in a quiet, sunny lab with classical music drifting in from the postdoc's office. As I walked into my first lab as a new graduate student, I was stunned to hear that the senior graduate student tuned in to soft rock. We younger students forged a plan: the first person to arrive could choose the radio station for the day, so we took turns sacrificing sleep to liberate ourselves from the tyranny of easy listening.

The situation eased when we split into two new laboratories. The young students in our new lab realized we now had control over our musical milieu. We pitched in for a cheap CD player and began our collection of morning, afternoon and late-night listening, including Canadian music to educate our exchange students.

My current lab chorale ranges from Finnish Internet radio, Brazilian love songs, silent CDs played on computers connected to headphones, and local rock stations, to the occasional pair of postdoc voices raised in chorus with the *Les Misérables* soundtrack. With research group members from all over the world, I expect nothing less than this cornucopia of fortuitous musical education. ■

Sidney Omelon is a PhD student in bone biomaterials at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.

BRICKS & MORTAR

Rensselaer Polytechnic Institute

The area around Rensselaer Polytechnic Institute in Troy, New York, is known for forging steel, not biomolecules. But this month, scientists and engineers started moving into a \$100-million biotechnology facility. Its founders hope that the building will become a biotech hub for upstate New York.

The centre, which received \$22.5 million from the state of New York, will contain four research constellations: biocatalysis and metabolic engineering, tissue engineering and regenerative medicine, systems biology, and biocomputation and bioinformatics. It will house 400 scientists in groups led by 50–60 principal investigators.

In her inaugural address as president of the institute five years ago, physicist

Shirley Jackson described biotechnology as “a field whose impact is so great, so full of promise, so well-suited to Rensselaer, that we simply must drive our stake into the ground of this new frontier.”

“Why not create a biotechnology institute for Rensselaer that would encompass fundamental research, industrial partnerships, technological innovation and undergraduate and graduate education?”

Today she adds: “There was a need to bring engineering ability to bear on the life sciences,” noting, for example, the importance of modelling and imaging technologies.

Last month, the facility that Jackson envisioned, the Center for Biotechnology and Interdisciplinary Studies, opened. Elias Zerhouni, director of the National Institutes of Health, and Bruce Alberts, president of the National Academy

of Sciences, were among those in attendance.

Since becoming chair of the biology department two years ago, centre co-director Robert Palazzo has recruited seven faculty members, and is still searching. There is stiff competition for good people in systems biology, tissue engineering and regenerative medicine, he says.

Different disciplines will mingle and collaborate in an open lab space. “The goal here is to enhance serendipity,” says Palazzo. The labs will have no walls: biologists, physicists and chemical engineers will be working side by side.

The set-up will test researchers’ willingness to share data. “If we get to the point where we covet information, or we hide information for our personal gain, then maybe we don’t belong in that building,” Palazzo says. ■

Eugene Russo is a freelance writer in Takoma Park, Maryland.

♦ www.rpi.edu/research/biotech

MOVERS

David Lane, executive director, Institute of Molecular and Cell Biology, Singapore



Following his first postdoc at the Imperial Cancer Research Fund in London, David Lane earned a rare freedom. He was offered a lectureship at Imperial College London, but was allowed a leave of absence to do another fellowship first at Cold Spring Harbor Laboratory in New York, on the understanding that he would return to Imperial after two years.

Almost 30 years later, Lane finds himself in a similar position. He is leaving the University of Dundee, UK, for two

years, to head the Institute of Molecular and Cell Biology in Singapore’s ‘Biopolis’ — an ambitious attempt by the Singapore government to build life sciences from the ground up.

The first time he went away with no worries about his return, good things happened. The freedom in New York “allowed me to be quite bold and take on things I wanted to”, Lane says. As a result, he was the first person at Cold Spring Harbor to make monoclonal antibodies — a key tool that would later fuel the biotech boom. And that independence, fostered early by his PhD adviser Avron Mitchison at University College London, set the pattern for his career. “Avron never put his name on anything I wrote,” says Lane, “he just let me get on with it.”

That ethos of independence allowed Lane to return to London with experience when he was 27. He already had his own grants, and so had funding

freedom. That allowed him to apply immunochemistry to identify and characterize proteins. As a result, he made key findings on the machinery of p53, a tumour-suppressor gene, and he has studied the gene and protein for much of his career.

Lane says that more young scientists should experience the freedom he had early in his career. “I think everybody needs it,” he says. But he says young scientists also can’t expect someone to hand them autonomy on a platter — they shouldn’t “wait for people to tell them what to do”, he notes.

Although Singapore has a reputation for top-down control, Lane says he expects to experience autonomy when he moves to the island nation in January. And the fact that he will return to his job in Britain in two years’ time will allow him to be typically bold in pursuing it. ■

CV

1990–2004: Personal chair in molecular oncology, University of Dundee, UK

1985–90: Senior staff scientist, Imperial

Cancer Research Fund

1977–85: Lecturer, Imperial College London

1978–80: Robertson research fellow, Cold Spring Harbor Laboratory, New York

1976–77: Postdoc, Imperial Cancer Research Fund, London